University of Alberta

ANALYSIS OF CONTINUOUS AND INTERVAL EXERCISE AND RECOVERY

by

William John McGarvey ©

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requirements for the degree of Doctor of Philosophy

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Traditionally, long duration, moderately intense exercise has played an important role in the attempt to manage body weight for the general public and athletes involved in non-endurance sports. Results of recent research suggests that higher intensity exercise may be more effective at controlling weight. Cross-sectional studies have shown that men and women who have participated in intense physical activity displayed lower fat percentage, reduced subcutaneous adiposity, and lower waist-to-hip ratio than those who did not (18;22). The importance of higher intensity exercise to weight loss has also been demonstrated by controlled training programs. Bryner et al. (3) found that 16 weeks of higher intensity exercise resulted in greater decreases in body fat than lower intensity exercise. Grediagin (6) had 12 untrained women perform 300 kcal of work using either a high intensity (80% VO_{2max}) or low-intensity (50% VO_{2max}) exercise program for 12 weeks. Both groups lost equal amounts of body fat, however, the high intensity group gained significantly greater muscle mass. High intensity exercise has been shown to increase exercise energy expenditure by decreasing exercise efficiency, increasing muscle mass, increasing resting energy expenditure, and improving fitness (7).

Continuous high-intensity exercise has been shown to be difficult for the average person to tolerate, resulting in increased levels of fatigue and anxiety compared to low to moderate exercise intensities (5). Interval exercise, a form of training consisting of periods of high intensity exercise separated by periods of rest or lower intensity, makes high-intensity exercise more endurable (7). In addition, the interval format, by incorporating periods of rest and recovery, allows for the performance of more minutes of high intensity exercise than the continuous format, providing for the possibility of greater physiological adaptation (17). These psychological and physiological benefits may make interval exercise an important adjunct to achieving increased levels of exercise intensity and the resulting changes in body composition (7).

Two recent studies have investigated the effect of interval exercise on body composition. Snyder et al. (15) found that a training program utilizing moderate intensity intermittent training resulted in increased aerobic capacity and reduced fat weight in 7 of 13 subjects. Tremblay et al. (19) demonstrated that high intensity interval exercise training resulted in significantly greater decreases in subcutaneous skinfold measurements than lower intensity continuous training. These positive changes occurred even though approximately twice as much energy was expended during the continuous program compared to the interval one.

Although these investigations indicate a role for interval exercise as an effective weight loss tool, there is little understanding of the mechanisms involved. The fact that the greater reductions in skinfolds occurred in Tremblay's investigation despite lower energy expenditure during exercise would seem to indicate that the benefits of interval exercise to body composition occurred post-exercise. Two possible post-interval exercise occurrences may have been responsible: increased post-exercise metabolism or a shift in post-exercise substrate utilization.

Interval exercise may result in a greater post-exercise metabolic rate as indicated by increased oxygen consumption. This increased metabolic rate may be of a duration and magnitude great enough to cause a significant reduction in adipose tissue stores (12;14). Recent investigations have supported the notion that higher intensity exercise results in greater post-exercise oxygen consumption compared to continuous exercise (1;2;9;23). Another possibility is that interval exercise promotes greater post-exercise utilization of fat as a substrate. Subsequent reductions in adipose tissue stores would therefore be the result of greater post-exercise fat utilization even if total caloric utilization was equal. In support of this hypothesis, fat utilization during recovery has been shown to increase proportional to exercise intensity (11).

The ability of exercise to affect substrate utilization may be of importance in weight loss. Recent investigations have demonstrated that low fat oxidation rates are present in obese and post-obese subjects and are a predictor of weight gain (4;13;20). Exercise may not only cause alterations in fat oxidation, but may also result in changes at the adipocyte. Lipolytic responsiveness has been shown to be enhanced in subjects with low body fat and/or trained athletes (8) and decreased in obese subjects or untrained controls (21). In support, McCarty (10) stated that the chief benefit of exercise in relation to weight loss may not be the expenditure of calories, but the ability to promote fat oxidation. However, a clear understanding of the type of exercise which best promotes increases in post-exercise fat oxidation has not yet been determined (16).

The purpose of this research was to investigate metabolic responses during and after high-intensity interval and moderate-intensity continuous exercise to determine if exercise intensity may be related to the promotion of long-term changes in body composition. This was accomplished by the integration of information from two distinct studies. The first study examined the effect of interval and continuous exercise on whole body post-exercise oxygen consumption and substrate utilization. This investigation supplied information regarding the difference in the magnitude of energy utilization following interval and continuous exercise and general information on substrate utilization. The second investigation more closely examined the effect of interval and continuous exercise on substrate utilization during exercise and recovery. This was accomplished by the measurement of specific blood borne fat and carbohydrate substrates, muscle glycogen, and the activity of key enzymes in the metabolic pathways of these substrates. The combined information from these two investigations provides a greater understanding of the role of higher-intensity exercise in the promotion of weight loss.

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CHAPTER 2

GENERAL REVIEW OF LITERATURE

2.1 Post-Exercise Oxygen Consumption

Research on post-exercise oxygen consumption dates back to the early 1900's when A.V. Hill and associates (53;54) formulated the classic "O_2 debt" theory. At the initiation of submaximal exercise, it was noted that a delay occurred in the attainment of a steady-rate VO_2. The difference between the attained steady-rate VO_2 and the actual VO_2 prior to the attainment of steady-rate VO_2 was referred to as the "oxygen deficit". The "oxygen debt" referred to O_2 consumption above baseline levels following exercise. The oxygen debt was deemed necessary to repay the O_2 deficit and was defined as the total amount of oxygen used after cessation of exercise in recovery there from in excess of an assumed or measured resting VO_2 (39).

These authors believed that oxygen consumption post-exercise was most closely tied to the removal of lactate. Hill suggested that 1/6 to 1/5 of the lactate was oxidized, thus supplying the energy required to convert the remaining portion of lactate into glycogen (53;54). These authors also noted that post-exercise oxygen consumption occurred in two phases: a rapid phase occuring in the first few minutes when oxygen consumption falls dramatically, and a slow phase, when oxygen consumption slowly returned to baseline/pre-exercise levels. Hill attributed the fast phase to the oxidation of lactate in the muscles where it was formed and the slow phase to the oxidation of lactate in muscles other than where it was formed. Margaria et al. (82) noted that the decline in blood lactate did not occur until after the fast phase, and therefore referred to the fast phase of oxygen debt as the "alactacid" phase. They further suggested that this phase was a result of the replacement of phosphogen stores. Margaria et al. concurred with Hill, also determining that the conversion of lactate to glycogen occurred during the slow phase and referred to this phase as the "lactacid" phase.

Later investigations by Bang (8) disputed the causal effect of lactate on the slow phase. Bang demonstrated that, during exercise, lactate levels could return to near-resting levels, however, there always occurred a slow phase of oxygen consumption (8). These results could not be reconciled with the idea that lactic acid determined oxygen consumption post-exercise. The lack of concrete evidence demonstrating a causal effect of lactate on post-exercise oxygen consumption, and the initiation of other investigations indicating that alternative mechanisms may be involved, resulted in Gaesser and Brooks (39) suggesting the term O_2 debt be replaced with a more inclusive term: excess post-exercise oxygen consumption (EPOC).

2.1.1 EPOC Methodologies

Although there have been numerous papers published on EPOC, comparing results can be difficult. Differences in methodology present a significant problem. An important component of all EPOC studies is the identification of EPOC termination: at what point is post-exercise oxygen consumption no longer greater than resting/baseline levels? Past investigations used a number of methods to determine EPOC termination. Some investigations have terminated EPOC when post-exercise oxygen consumption fell within 1 standard deviation of the mean pre-exercise VO_2 for two consecutive minutes (28;94). Others terminated EPOC when the 5-min average of recovery VO_2 equaled mean pre-exercise VO_2 (126;128;160). Termination in some investigations took place at specific times (generally 1-h post-exercise), regardless of whether or not resting values had been reached (3;15;20) This lack of consistency in the identification of EPOC termination makes the comparison of data less than ideal.

Another issue involves the degree to which continuous oxygen consumption has been measured. Many investigations measured post-exercise oxygen consumption only periodically (generally for a few minutes per hour) as opposed to continuously. Periodic measurements were then used to calculate total oxygen consumption. Investigations have generally used periodic monitoring when the post-exercise period is in excess of 1-h (4;6;7;137). Although this technique is most likely necessary for subject comfort, etc., varied approaches to measuring EPOC increase the difficulty of clearly understanding and comparing results.

2.1.2 Components of EPOC

It is generally accepted that EPOC consists of two components: fast and slow. The two components generally form a double-exponential curve, which demonstrates a rapid decrease in oxygen consumption followed by a slower decrease down to resting levels. Frey et al. (34) defined the rapid phase as the first 10-min post-exercise and the slow phase as the time from 10-min post-exercise until the termination of EPOC.

2.1.3 EPOC and Training

Limited research does suggest differences in EPOC related to the training level of the subject. Frey et al. (34) investigated the differences in EPOC between trained and untrained subjects after 300 kcal of exercise at 80% and 65% VO_{2max}. EPOC duration for untrained subjects was greater than 60 min post-exercise following both treatments, while

EPOC duration for trained subjects was less than 50 min. Despite this difference in EPOC duration, there was no difference in EPOC magnitude between trained and untrained subjects for the 65% VO_{2max} treatment. At 80% VO_{2max} however, untrained subjects demonstrated a greater duration and magnitude compared to trained subjects. Sedlock (126) investigated five endurance-trained and five sedentary males after 300 kcal of exercise at 50% VO_{2max}. EPOC duration was slightly longer for the sedentary males, however, EPOC magnitude was not different. The results of these investigations seem to indicate that the training status of subjects will affect the duration of EPOC but will not have a significant effect on magnitude.

2.1.4 Metabolic Basis for EPOC

A number of factors are believed to be involved as mechanisms for EPOC. These include: replenishment of oxygen stores (myoglobin and hemoglobin), increased ventilation, circulation and body temperature, resynthesis of phosphogens (ATP and phosphocreatine), residual effects of hormones, and lactate removal (13). It is suspected that the mitochondria, the site of oxygen consumption within the cell, may be closely tied to these mechanisms. Gaesser and Brooks (39) stated that the post-exercise environment may contribute to the increased function of mitochondria. Many mechanisms are suspected to be most prominent in the first hour post-exercise (39). However, extended EPOCS (4-12 h) have been recorded. The mechanisms suspected to have the greatest effect on long term EPOC are an increased rate of energy-requiring triglyceride fatty-acid futile cycling and a shift from carbohydrate to fat as substrate source (13).

2.1.4.1 Temperature and EPOC

Of the possible mechanisms of EPOC, temperature has been most extensively investigated. In a review of EPOC, Gaesser and Brooks (39) stated that temperature is perhaps the most important factor contributing to the elevated post-exercise VO_2. Generally, investigations have found end-exercise core temperature to be positively related to exercise intensity (7;34). Two human studies support the relationship between EPOC and increased temperature. Frey et al. (34) found rectal temperature to correlate highly with EPOC duration ($r = 0.89$) and Chad and Wenger (19) found temperature to have a low ($r = 0.64$) to moderate ($r = 0.74$) correlation with EPOC.

Most other studies, however, reported a weaker relationship between temperature and EPOC (7).(80) Two investigations (6;109) found significantly greater EPOC magnitudes with increased duration of exercise at 70% VO_{2max}, but with no significant difference in post-exercise core temperature. Bahr et al. (6) found an exponential increase in EPOC related to exercise intensity, yet no significant difference in post-exercise temperature, which decreased rapidly and reached control values in less than 1-h. Most investigations that found a weak relationship between EPOC and temperature utilized extended EPOC measurement periods (3-24h) in which subjects were fed during the post-exercise period. Investigations that reported shorter duration EPOC found a stronger relationship with temperature. As stated earlier, differences in methodology make comparisons of EPOC results somewhat difficult. These differences make it difficult to determine the exact relationship between EPOC and temperature.

2.1.4.2 Lactate and EPOC

Although early research linked EPOC strongly to post-exercise lactate removal, little recent research has been performed in this area. Gaesser and Brooks (39) stated in their review of EPOC that post-exercise lactate removal is slow and remains elevated long after EPOC is terminated. Contrarily, Frey et al (34) exercised trained and untrained subjects for 300 kcal at 65% and 80% VO_{2max} and found that untrained subjects achieved higher end-exercise lactate levels during submaximal exercise than did trained subjects. When related to EPOC, they observed a high correlation ($r = 0.90$) between blood lactate levels and EPOC.

2.1.4.3 Substrate Cycling and EPOC

Bahr et al. (5) determined the rate of triglyceride/fatty-acid cycling (TG/FA cycling) by calculating the difference between the rates of fatty-acid mobilization and oxidation. Bahr et al. (5) concluded that the energy cost of the increased TG/FA cycling may account for as much as half of the slow component of EPOC. It was calculated that substrate cycles would only have to work at 3-17% of maximal capacity to account for an oxygen uptake of approximately 1 ml O_2/min/kg, an increase of approximately 30-40 ml O_2/min/kg in a 70 kg man (4). A recent investigation by Mulla et al. (90) questions the importance of TG/FA cycling post-exercise. Mulla et al. performed catheterization of the subcutaneous vein on the abdominal wall, the femoral vein and the radial artery. In addition, blood flow was measured using the ultrasound/Doppler technique. This process allowed the release and utilization of non-esterfied fatty acids (NEFA) to be measured for 3-h following exercise performed at 40% and 60% VO_{2max}. The investigation concluded that post-exercise lipid mobilization and oxidation are tightly matched up to 3-h post-exercise, and that there appears to be little evidence of significant TG/FA cycling.

2.1.4.4 Substrate Utilization and EPOC

A shift in post-exercise substrate utilization from carbohydrate to fat would result in greater oxygen consumption to provide equivalent energy. One liter of oxygen will provide 5.05 kcal of energy when carbohydrate is the source, whereas only 4.70 kcal is provided with fat. A shift towards increased fat oxidation would result in an increase in oxygen consumption to provide equivalent amounts of energy. Chad and Wenger (19) exercised subjects for 30, 45 and 60 min at 70% VO_{2max} and reported that the correlation between RER and EPOC accounted for 74 to 79% of the variance in EPOC magnitude.

Higher intensity exercise has been shown to result in higher RER during exercise than low to moderate intensity exercise (78). Christmass et al. (25) compared high-intensity intermittent exercise and moderate intensity continuous exercise and noted that fat oxidation was almost 3.0 times lower and carbohydrate oxidation 1.2 times higher during the intermittent exercise. These results are supported by Bahr et al. (7), who exercised subjects for 80 min at three different intensities (29%, 50%, 75% VO_{2max}) and found a positive relationship between exercise intensity and RER during the last minute of exercise. Post-exercise, high intensity exercise has been shown to result in greater fat utilization, and therefore a lower RER than lower intensity exercise (103). Laforgia et al. (78) compared continuous exercise (30 min at 70% VO_{2max}) to intermittent exercise (2-min at 105% VO_{2max} separated by 2-min rest) and found no significant difference between baseline RER and post-exercise RER values for continuous exercise. However, post-exercise RER values following intermittent exercise were significantly lower than baseline levels.

Care must be taken when interpretating RER values (VCO_2/VO_2) as a direct indicator of substrate utilization. Production of carbon dioxide (CO_2) occurs within cells (metabolic CO_2) due to fuel oxidation, and in blood as a result of hydrogen ion buffering (non-metabolic CO_2). Higher intensity, non-steady state exercise results in the production of both metabolic and non-metabolic CO_2 with RER values therefore exceeding the 1.0 associated with pure carbohydrate oxidation (33). During recovery from exercise, factors other than substrate utilization affect RER values. Hydrogen ion buffering during exercise utilizes bicarbonate ions and the replenishment of these ions during recovery requires carbon dioxide. Laforgia et al. (78) has stated that this utilization or "retention" of CO_2 results in a lower RER then that which would accurately reflect the current substrate utilizaiton. In addition, during recovery, oxygen is required to restore myoglobin and hemoglobin to normal saturation levels (13). This increased oxygen uptake not utilized directly for fuel oxidation would also result in a lower RER than would be expected based on substrate utilization.

2.1.4.5 Catecholamines and EPOC

Catecholamine concentrations are related to exercise intensity with low to moderate intensities resulting in almost no increase in catecholamines and intensities beyond 50-70% VO_{2max} demonstrating exponential increases (34). Exercise intensity is also positively related to post-exercise catecholamine levels. Frey et al. (34) exercised subjects at 65% and 80% VO_{2max} for 300 kcal and concluded that nor-epinephrine and sympathetic activity play an important role in the fast component and initial aspect of the slow component of EPOC. However, they concluded that the return of nor-epinephrine to resting levels before the termination of EPOC suggests that other factors are responsible for the extended phase of the slow component of EPOC.

2.1.5 Exercise Duration and EPOC

Research generally supports the positive relationship between exercise duration and EPOC. However, this relationship seems to be strongly affected by exercise intensity. Gore and Withers (42) found no increase in EPOC when subjects exercised for 20, 50 and 80 min at 30% VO_{2max}, but did find a positive linear relationship for the three duration's at 50% VO_{2max}. This relationship appears stronger at higher intensities. Five investigations exercised subjects at 70% VO_{2max} for various durations (3;19;42;81;109). Chad and Wenger (19) utilized durations of 30, 45, and 60 min and found an exponential increase in EPOC duration. Three other investigations using similar durations found a linear increase, but not an exponential increase. Only Maresh et al. (81), who exercised men at 70% VO_{2max} for 20 and 40 minutes, did not find an increase in EPOC magnitude with increased duration at 70% VO_{2max}. These findings suggest that a weak relationship between EPOC and duration exists at lower intensities, but as intensity increases (in the range of 70%) a positive linear relationship appears to exist.

2.1.6 Exercise Intensity and EPOC

Although results are equivocal, research supports a positive relationship between exercise intensity and EPOC. Only one investigation found a negative relationship. Chad and Quigley (20) reported that exercising subjects for 30 min at 50% and 70% VO_{2max} resulted in a greater EPOC magnitude following exercise at 50% than at 70%. Two investigations found no relationship. Sedlock et al. (125) had active young women cycle at 40% and 60% VO_{2max} for 850 kJ and found no significant difference in EPOC magnitude. Kaminsky et al. (69) matched work with subjects walking at 35% VO_{2max} for 60 min, and running at 70% VO_{2max} for 30 min and also found no significant difference in EPOC. However, most studies support a positive relationship. Dawson et al. (28) exercised eight healthy young women at 65% VO_{2max} for 30 min and also at 55% and 45% VO_{2max} with duration set to match work. They found EPOC magnitude to be significantly greater after 65%, but no significant difference between 55% and 45% was reported. Smith et al. (129) exercised eight trained men and women for 30 min at 40%, 50%, and 70% VO_{2max} and found a linear increase in EPOC magnitude related to intensity. Three investigations found exponential increases in EPOC due to intensity. Brehm (15) had subjects walk/run for 3.2 km at four different speeds (3.2, 6.4, 8.1 and 11.3 $km.h^{-1}$) and found an exponential increase in EPOC magnitude related to intensity. Bahr et al. (7) also found an exponential increase when subjects exercise for 80 min at 29%, 50% and 75% VO_{2max}. The strongest evidence for the impact of intensity on EPOC was produced by Gore and Withers (42). In their investigation, each subject completed nine exercise treatments consisting of 20, 50 and 80 min of treadmill exercise at 30%, 50% and 70% VO_{2max}. EPOC magnitudes were not significantly different for the three durations at 30% VO_{2max}. However, there were significant increases related to both duration and intensity at 50 and 70% VO_{2max}. In conclusion, Gore and Withers stated that exercise intensity was the major determinant of EPOC.

This positive relationship between EPOC and exercise intensity is further strengthened by analysis of investigations using intensities greater than anaerobic threshold. Laforgia et al. (78) matched work for continuous (30 min at 70% VO_{2max}) and intermittent high-intensity (20 x 1 min at 105% VO_{2max} with intervening 2-min rest) treadmill exercise and found significantly greater EPOC magnitude following the higher intensity exercise (15.0 ± 3.31 to 6.9 ± 3.8 liters). With a similar protocol Treuth et al. (137) found significantly greater post-exercise metabolic rates (5.94 ± 0.58 to 5.48 ± 0.53 kJ/min) following high intensity intermittent exercise (15 x 2-min at 100% VO_{2max} with intervening 2-min rest) compared to lower intensity continuous cycling (60 min at 50% VO_{2max}). Comparing two high intensity treadmill exercise treatments, one continuous (10 min at 81.1% VO_{2max}) and one intermittent (2 min at 89.2% alternated with 2 min walks at 93.8 m/min), Brockman et al (16) found significantly greater EPOC following the slightly higher-intensity intermittent exercise. In a study utilizing high-intensities more likely achievable by recreational athletes, Ziegenfuss and Sedlock (160) compared continuous (65% VO_{2max}) and interval (2-min at 40% VO_{2max} followed by 2-min at 90% VO_{2max}) exercise matched for work (25,000 kpm) and found significantly greater EPOC magnitude following interval exercise.

Research demonstrates that exercise intensity appears to have a strong effect on EPOC magnitude. This effect appears to require a threshold intensity of 50% VO_{2max} (42). Moderate intensities from 50% VO_{2max} to near anaerobic threshold result in an increase in EPOC magnitude, with an exponential relationship occurring for intensities from near anaerobic threshold to above VO_{2max}.

2.1.7 Summary of Post-exercise Oxygen Consumption

The review of literature on post-exercise oxygen consumption strongly implies that high intensity exercise results in an increase in EPOC magnitude greater than what would occur following low to moderate intensity continuous exercise. However, a clear understanding of post-exercise energy utilization following interval vs. continuous exercise has yet to be achieved. A number of factors contribute to this lack of understanding, including exercise protocols using unmatched work and/or duration for the interval and continuous programs, non-continuous measurement of EPOC, and post-exercise feeding. In addition, past investigations utilized intensities exceeding VO_{2max} and durations greater than the 30-min duration recommended for health and fitness benefits, thus reducing the applicability of the results to the average person using exercise as a weight-loss tool. Addressing these issues would assist in understanding the mechanisms by which interval exercise affects body composition and weight loss.

2.2 Exercise and Recovery Substrate Utilization

As stated in the preceding sections, the effect of interval exercise on weight loss may occur through an increase in post-exercise oxygen consumption and energy utilization. Another possible mechanism would be a shift towards the utilization of fat as a post-exercise substrate. Past investigations indicate that the primary fuels utilized for muscle contraction are fats and carbohydrates. Branch chain amino acids are available as fuels, although their contribution is thought to be negligible (65). Therefore, this review will focus primarily on the metabolism of fats and carbohydrates during exercise and recovery. Primary carbohydrate metabolic pathways and substrates will be reviewed first, followed by a review of fat utilization.

2.2.1 Carbohydrate Pathways and Substrates

Extensive research over the past decades has produced a relatively clear understanding of carbohydrate utilization during exercise. However, the role of fats during exercise, and the role of both fats and carbohydrates in the post-exercise period is not as well understood (65). The interrelationship between the two substrates are also not clearly understood, however, the main factor determining the degree of fat or carbohydrate utilization during exercise appears to be exercise intensity (66). It is suspected that this is primarily a result of the difference in rate of ATP production between the two substrates. When blood-borne FA are oxidized, the maximum rate of high-energy phosphate (HEP) formation is approximately 0.40 mol HEP/min. However, the aerobic or anaerobic breakdown of glycogen or glucose can generate approximately 1.0 – 2.4 mol HEP/min (65).

The two main metabolic pathways for carbohydrate utilization within the muscle are glycolysis and glycogenolysis with one result being the production of Pyruvate. Pyruvate has three fates. It can be acted upon by the enzyme lactate dehydrogenase (LDH) and converted to lactic acid, it can be acted upon by the enzyme pyruvate dehydrogenase (PDH) and converted to acetyl-CoA, or it can be acted upon by a transamination enzyme and, by accepting an amino group from the amino acid glutimic acid, it can convert pyruvate to alanine (65). The first two fates, conversion to lactic acid or acetyl-CoA, are the main fates of pyruvate (58). The factors determining whether pyruvate is converted to acetyl-CoA or lactic acid are the rate of pyruvate formation (glycolytic flux), the cytosolic redox state (NADH/NAD), the number and size of mitochondria, and the availability of oxygen (58). PDH is intricately involved in carbohydrate utilization as it controls the flux of pyruvate-derived acetyl-CoA into the tricarboxylic acid (TCA) cycle (108). Therefore, understanding PDH activity will provide a greater understanding of carbohydrate utilization during rest, exercise and recovery.

2.2.2 PDH Structure and Function

The structure, function and regulation of PDH have been reviewed in recent years (148). PDH is a multi-enzyme complex, located within the mitochondrial inner membrane (MIM), that catalyzes the pivotal irreversible reaction leading to the consumption of glucose in aerobic, energy-generating pathways. PDH catalyzes the oxidation and decarboxylation of pyruvate to acetyl-CoA. The PDH reaction can be summarized as follows:

$$\text{Pyruvate} + \text{NAD}^+ + \text{CoASH} \xrightarrow{\text{PDH}} \text{acetyl-CoA} + \text{NADH} + \text{H}^+ + \text{CO}_2$$

PDH is a multi-enzyme complex composed of three main catalytic subunits: pyruvate decarboxylase (E1), dihydrolipoamide acetyl-transferase (E2), and dihydrolipoamide dehydrogenase (E3). Closely associated with these subunits are multiple copies of two regulatory enzymes: PDH-phosphatase and PDH-kinase. The E1, E2 and E3 subunits are the major catalytic components in the 5-step process to remove the alpha-terminal carboxyl group from pyruvate and direct the resulting acetyl-group to a free coenzyme-A (CoASH), with the resulting acetyl-CoA product being oxidized in the tricarboxylic acid cycle (TCA). In step 1, E1 catalyzes the decarboxylation of pyruvate. Step 2 requires E1 to catalyze the reductive acetylation reaction yielding an acetyl group covalently linked to E2. E2 then catalyzes the transfer of acetyl groups to CoASH to form acetyl-CoA in step 3. In steps 4 and 5, E3 mediates the transfer of electrons from E2 to nicotinamide adenine dinucleotide (NAD), converting it to its reduced form (NADH + H^+).

2.2.3 PDH Regulation

The phosphorylation-dephosphorylation cycle is catalyzed by two regulatory subunits non-covalently bound to the PDH complex: PDH-phosphatase and PDH-kinase. PDH-phosphatase hydrolyzes monophosphate esters from specific serine residues at three distinct sites on E1, resulting in the transformation of PDH from its inactive form (PDH_c) to its active form (PDH_a). PDH-kinase has the opposite effect, reesterfying these sites in a sequential fashion (1-3) with a resulting reversal of the transformation and varying degrees of inactivation and resistance to reactivation (108).

PDH-kinase and PDH-phosphatase are controlled by a number of allosteric modulators, including the mitochondrial ratios of NADH/NAD, acetyl-CoA/CoASH, and ATP/ADP. These ratios are a reflection of the intra-mitochondrial balance between substrate supply, tissue oxygen availability and cellular ATP demand (104). For example, during periods of low energy requirements within the cell, NADH will accumulate within the mitochondria, resulting in an increase in the NADH/NAD ratio, activation of PDH-kinase, and inhibition of PDH. As the energy needs of the cell increase, NADH will be oxidized to NAD, reducing the NADH/NAD ratio and stimulating PDH-phosphatase. These ratios then assist in determining the flux of carbohydrate derived acetyl-CoA through the TCA cycle and the ATP production that occurs as a result.

The NADH/NAD ratio functions in two ways to regulate PDH: allosteric regulation and end product inhibition. Allosteric regulation occurs as NADH accumulation inhibits PDH-phosphatase and increases the activity of PDH-kinase with a resulting transformation of active PDH_a into inactive PDH_c. Alternately, NAD accumulation inhibits PDH-kinase and activates PDH-phosphatase. End-product inhibition occurs when increasing amounts of NADH displaces NAD from its binding site on the E3 subunit of PDH, thus reducing the conversion of PDH_c to PDH_a.

Changes in the acetyl-CoA/CoASH ratio affect PDH_a activity and transformation in a similar allosteric manner to NADH/NAD. In addition, acetyl-CoA also functions with an end-product inhibition by reversing the step involved in the transfer of the acetyl-group to CoASH, thereby halting any further production of acetyl-CoA. Acetyl-CoA and CoASH are important metabolites in controlling PDH activity. When the rate of acetyl-CoA generation from carbohydrate and/or fat sources exceeds its ability to condense with oxaloacetate to form citrate, acetyl-CoA accumulates as a result. Increased acetyl-CoA results in a reduction of CoASH. To compensate for this imbalance, acetyl-CoA will have the CoA stripped off and replaced with a carnitine, converting it to acetyl-carnitine. Accumulating acetyl-carnitine is then exported from the inner mitochondria by the enzyme carnitine acyl-carnitine translocase (73). This allows for the maintenance of a mitochondrial pool of CoASH available for oxidative metabolism and the restriction of mitochondrial CoASH depletion (107).

The ATP/ADP ratio has an effect that is confined to only one of the regulatory subunits, PDH-kinase. ATP is a substrate for, and ADP is a product of, PDH-kinase. Therefore, a rise in the ATP/ADP ratio results in a greater accumulation of substrate and a reduction in product for PDH-kinase, with a resultant increase in PDH-kinase activity. Continued high ATP/ADP ratios, and the consequent increase in PDH-kinase activity, result in PDH_c becoming sequentially phosphorylated on the three E1 sites. This situation makes it increasingly difficult to remove phosphate groups by PDH-phosphatase, and therefore requires a more potent PDH-phosphatase stimulation, or alternatively a greater inhibition of PDH-kinase, for there to be an increase in PDH_a accumulation.

Three other metabolites play a role in the activation of PDH: pyruvate, the hydrogen ion (H^+), and calcium (Ca^{2+}). Pyruvate exerts its effect through substrate binding and allosteric regulation. Pyruvate is the principle substrate for PDH, and by its presence it ensures substrate binding to promote adequate PDH_a flux. Allosterically, increases in pyruvate concentration have an inhibitory effect on PDH-kinase. It has been speculated that increased H^+ increases activation of PDH_a through several mechanisms, including altered metabolite affinity resulting in greater PDH-phosphatase activity and reduced PDH-kinase activity, and increased substrate and allosteric affinity for pyruvate (104). Calcium is involved through its activation of PDH-phosphatase. Increased calcium in the mitochondria is a result of a sustained increase in cytosolic calcium ions, and their entry into the mitochondria due to an imbalance in ionic calcium concentrations between extra-mitochondrial and intra-mitochondrial spaces (58). Therefore, activation/deactivation of PDH is affected by both the metabolic state and the contractile state of the muscle fiber (58).

The basic premise for the regulation of PDH was first proposed by Randle et al. and referred to as the glucose-fatty acid cycle (111). Randle proposed that increased fat oxidation exerted an effect at the level of PDH which resulted in reduction of intramuscular glucose oxidation. Increased oxidation of fat results in a rising intra-mitochondrial ratio of acetyl-CoA/CoASH and NADH/NAD. Rising levels of these ratios inhibit the transformation of PDH_c to PDH_a. In addition, increased fat oxidation results in the accumulation of intra-mitochondrial citrate, which migrates out of the mitochondria to increase cytosolic levels of citrate. These increased ratios reflect an abundance of substrate for the TCA cycle and electron transport chain (58). Increased cytosolic citrate inhibits phosphofructokinase (PFK), a rate-limiting enzyme of glycolysis. The reduced activity of PFK results in the accumulation of glucose-6-phosphate (G-6-P) which in turn inhibits hexokinase (HK), restricting glucose uptake and glycogen utilization (107). The overall result of these many reactions is that increased fat oxidation resulted in the inhibition of carbohydrate metabolism at key enzymatic sites and therefore reduced carbohydrate utilization.

Recent studies support the function of the glucose-fatty acid cycle at rest, however, other factors appear to be involved during exercise. Following high carbohydrate feeding, Putman et al. (108) noted that during exercise, high levels of PDH_a occurred despite the acetyl-CoA/CoASH ratio increasing five-fold from rest and a two-fold increase in citrate concentration. These results call into question the regulatory effect of acetyl-CoA and citrate on carbohydrate metabolism during exercise. Recent research by Spriet et al. (132) suggests that calcium may be the primary factor regulating PDH_a transformation during exercise.

2.2.4 PDH and Exercise

Odland et al. (100) cycled subjects at three levels of exercise intensity (35%, 65%, and 90% VO_{2max}) and demonstrated that the level of PDH activation was positively correlated with exercise intensity. Howlett et al. (59) used a similar protocol (10-min exercise at 35%, 65% and 90% VO_{2max}) and found that PDH transformation was directly related to increasing power output and the calculated flux through the enzyme. PDH_a activity increased above resting levels (0.40 ± 0.08 to 0.65 ± 0.18 mmol/kg wet muscle/min) after 1-min of exercise at all three power outputs, with 65% and 90% being higher than 35%. PDH_a activity at 35% (~1.5 mmol/kg wet muscle/min) and 65% (~2.7 mmol/kg wet muscle/min) did not change from 1 to 10 min, whereas it increased at 90% (~3.3 to 4.0 mmol/kg wet muscle/min). These results demonstrate that PDH is quickly activated during exercise and that the level of activation is related to intensity.

In regards to high-intensity intermittent exercise, Putman et al. (105) investigated the activation of PDH during 3 bouts of 30 s maximal isokinetic cycling separated by 4-min recovery periods. Resting levels of PDH activation were 14% (0.45 ± 0.15 to 0.65 ± 0.18 mmol/kg wet muscle/min). After 6 s of maximal exercise PDH_a was activated to 48%, and was almost completely activated (95% activation) at 15 s. Before the third bout PDH_a activation was at 42% and increased to almost complete activation by 6 s of activity. These results demonstrate that PDH is almost completely activated during high intensity exercise, and that with repeated bouts, the level of PDH activation during the recovery phase increases.

Many investigations, particularly those measuring post-exercise oxygen consumption and energy utilization, are performed with subjects being required not to eat for at least 12 h before testing. This serves to eliminate the thermal effect of food on metabolism so that a valid resting baseline oxygen consumption rate can be determined. High-intensity interval exercise has been shown to deplete muscle glycogen stores in active muscle by approximately 70% (38). In addition, it is likely that liver glycogen stores would be severely reduced also, due to the lack of feeding for 12-h and the requirements of interval exercise. Therefore, following interval exercise in this food restricted state, the body may be in a state of carbohydrate deprivation. St. Amand et al.

(134) studied PDH activity at rest during carbohydrate deprivation brought on by a 6-day low-carbohydrate diet. In this state PDH activity (0.18 ± 0.04 mmol/kg wet muscle/min) was below the resting level reported in other investigations by Putman et al. (105) (0.45 ± 0.15 mmol/kg wet muscle/min) and Odland et al. (100) (0.40 ± 0.08 mmol/kg wet muscle/min). The results of this investigation suggest that post-exercise, in a carbohydrate-deprived state, PDH activity may be found to be below resting levels.

Two investigations have studied the effect of plasma non-esterfied fatty-acids (NEFA) on PDH activity. Stellingwerff et al. (135) reported that cyclists exercising at 55% VO_{2max} in a condition of reduced [NEFA] demonstrated increased RER and PDH activity compared to control. Increased PDH activity was determined to be due to a decrease in the NADH/NAD ratio. In contrast, under conditions of elevated plasma [NEFA], reduced PDH activity was related to higher mitochondrial NADH concentration (97).

2.2.5 PDH and recovery

Only one investigation could be found that investigated PDH activity during recovery without post-exercise feeding. Richards et al. (115) exercised rainbow trout to exhaustion and measured PDH during rest, exercise and 32-h of recovery. PDH values decreased from near maximal levels at the end of exercise to resting levels within 15-min post-exercise. Decreases in white muscle free carnitine, increases in fatty acyl-carnitine, and sustained elevations of acetyl-CoA and acetyl-carnitine indicated a rapid utilization of lipid, both IMTG and plasma NEFA, to supply ATP for recovery.

2.2.6 Conclusions Regarding PDH

The majority of research on PDH and exercise has utilized either moderate continuous exercise intensities or supramaximal intermittent intensities. The effect of high intensity submaximal interval exercise has not been explored. In regards to the activity of PDH during recovery from exercise, little research could be found. In addition, although research has been performed looking at various enzymes related to fat metabolism during recovery from exercise (37;155), it would seem to provide only a partial explanation of substrate utilization during recovery. A more complete understanding would be achieved by measuring key enzymes in both carbohydrate and fat pathways. Therefore, research on the activity of PDH during interval exercise and recovery would be beneficial.

2.2.7 Carbohydrates during Exercise and Recovery

PDH is the key enzyme in controlling the flux of carbohydrate based acetyl-CoA into the mitochondria (104). A number of recent reviews have covered carbohydrate metabolism during exercise (27;43;46;48-51;55;65). Carbohydrates are available as a fuel in three primary forms in the body: muscle glycogen, liver glycogen and blood glucose. The greatest amount of carbohydrates are stored within muscle, with about a quarter of that amount stored in the liver, and small amounts available in plasma as blood glucose (66). Muscle glycogen can be used directly as a fuel in skeletal muscle, whereas liver glycogen must first be converted to glucose by liver glycogenolysis, then released into the blood as glucose and taken up by the muscle before it can be utilized as a fuel. Other substrates, such as lactate, amino acids, or glycerol can be converted to glucose by gluconeogenesis in the liver and then released into the blood. Exogenous sources of carbohydrates are also available as fuels via absorption by the intestinal system (65).

The importance of carbohydrates to exercise is paramount as they predominate at moderate to high power outputs, with muscle glycogen and glucose utilization increasing exponentially to the relative work rate (116). In contrast, peripheral lipolysis appears to be stimulated maximally at lower intensities, with reduced release into plasma and uptake into active muscle with increasing exercise intensity (116).

Exercise intensity and duration affect carbohydrate utilization during exercise. As exercise intensity increases there is increased reliance on carbohydrates, and as exercise duration increases there is a decline in carbohydrate contribution and an increase in fat utilization (46). Post-exercise, based upon metabolic data, there is a distinct shift in carbohydrate utilization with a decreased utilization proportional to exercise intensity (103). The importance of carbohydrates to exercise is supported by research that demonstrates the sole use of lipids as a fuel source cannot usually support exercise intensities in excess of 50-60% of maximal oxygen uptake (47).

2.2.7.1 Muscle Glycogen during Exercise and Recovery

Investigating the effect of submaximal exercise on muscle glycogen utilization, Vollestad and Blom (143) reported that, at low intensities (43% VO_{2max}), depletion occurred in almost all type I fibers and only 20% of type IIa fibers. Moderate intensity exercise (61% VO_{2max}) resulted in depletion in all type I and 65% of type IIa fibers and high intensity exercise (91% VO_{2max}) resulted in depletion in all type I and IIa and 50% of type IIb fibers. Gollnick et al. (40) demonstrated that, as low to moderate intensity exercise is extended in duration, slow twitch fibers fatigue due to loss of glycogen and fast twitch fibers are recruited. At this point, glycogen depletion will increase in fast twitch fibers.

Gollnick et al. (40) demonstrated that glycogen depletion rates increased dramatically with increasing exercise intensity, with depletion rates being 2.7 and 7.4 times greater respectively at workloads demanding 64% and 84% VO_{2max} than at workloads requiring 31% VO_{2max}. Glycogen depletion rates have been found to increase in all fiber types at supramaximal intensities (144). However, the glycolytic capacity of type I fibers was 30-35% lower than the capacity of type II fibers, in agreement with differences in activity levels of the enzymes phosphorylase and phosphofructokinase within these muscle types. This was in agreement with Gollnick et al. (40), who found that at supramaximal intensities, glycogen depletion occurred first in fast twitch fibers.

Past investigations have indicated that high intensity interval or intermittent exercise results in significant decreases in muscle glycogen concentrations. Futre et al. (38) demonstrated that five sets of 90 s cycling at maximal intensity resulted in a 70% decrease in muscle glycogen concentration from resting levels (125.6 mmol/kg wet wt) to end-exercise (25.6 mmol/kg wet wt). MacDougall et al. (79) exercised subjects at 140% of maximal aerobic power for 1-min intervals (3-min rest periods between) until 30 s of exercise could not be maintained, and reported depleted glycogen concentrations of approximately 70%. Submaximal continuous exercise also results in muscle glycogen depletion, but at a drastically reduced rate. Hurley et al. (60) required 120 min of cycling at 65% VO_{2max} to reduce muscle glycogen by 70% (285 to 82 mmol/kg dry wt). Kiens et al. (74) had subjects perform knee extensions for 120-min and only depleted muscle glycogen by approximately 60% (393 to 160 mmol/kg dry wt).

Although glycogenolysis has been demonstrated to be activated very rapidly at the onset of maximal cycle exercise, the rate of glycogen utilization appears to diminish with successive bouts (68). Spriet (133) demonstrated that during repeated bouts of 30-s maximal exercise, the rate of glycogenolysis decreased and glycolysis and oxidative metabolism increased with successive bouts. In bout 3, the work performed was 82% of bout 2, whereas glycogenolysis was only 32% of bout 2. Further evidence of the diminishing contribution of glycogenolysis during repeated bouts of high intensity exercise was contributed by Trump et al. (138) who had subjects perform three 30 s bouts of maximal isokinetic cycling separated by four minutes of complete rest. Results indicated a diminishing contribution from the phosphogen system and glycogenolysis as each of these systems contributed only approximately 15% of the total ATP utilization during the third bout. These results suggested that aerobic metabolism of blood glucose becomes the dominant source of ATP provision during repeated sprinting (138).

In a review on glycogen resynthesis after different exercise intensities, Pascoe and Gladden (102) stated that typical rates of muscle glycogen resynthesis after short term, high intensity exercise (15.1 to 33.6 mmol/kg/hr) are much higher than glycogen resynthesis rates following prolonged exercise (~2 mmol/kg/hr), even when optimal amounts of oral carbohydrate are supplied (5-8 mmol/kg/hr). The increased rate of glycogen resynthesis following higher intensity compared to low or moderate intensity exercise occurs even if the degree of muscle glycogen depletion is similar following both exercise treatments (112).

The most likely reasons for the increased rate of resynthesis following high intensity exercise are: higher recovery blood glucose levels, increased recovery insulin levels, higher end-exercise levels of glucose and glycolytic intermediates, higher recruitment of type II fibers during exercise, and reduced activity of the enzyme phosphorylase during recovery (57;142). Blood glucose levels after short term high intensity exercise have been shown to increase to elevated levels (6.6 to 8.9 mmol/L) compared to the relatively stable euglycemic levels found during low to moderate intensity exercise (5.0 mmol/L) (102). These higher blood glucose levels post-exercise result in blood insulin levels that are approximately six times higher (~60U/ml) than after prolonged moderate intensity exercise (~10U/ml) (102). In addition, intra-muscular glycolytic intermediates, including muscle glucose (7-fold), glucose-1-phosphate (12-fold) and glucose-6-phosphate (30-fold), can be increased over resting levels after high intensity exercise (68) with the greatest increases in substrate concentrations occurring prior to the enzyme PFK (21). Vollestad et al. (143) reported faster resynthesis taking place in the first 90 min of recovery in type II fibers compared to type I. Brau et al. (14) noted that in recovery from high intensity exercise, there was not only a dramatic increase in the enzyme glycogen synthase, but also a significant reduction in the activity of glycogen phosphorylase. Bangsbo (9) concluded that in the post-exercise recovery period, muscle glycogen resynthesis following high intensity exercise has high metabolic priority, resulting in increased post-exercise lipid combustion despite a high carbohydrate intake.

2.2.7.2 Blood Glucose during Exercise and Recovery

The processes involved in controlling blood glucose utilization during exercise are examined in recent reviews (51). During resting conditions, the principle role of liver glycogen is to maintain constant blood glucose levels (euglycemia). At rest, skeletal muscle accounts for approximately 15-20% of total peripheral glucose utilization. At moderate exercise intensities (55-60% VO_{2max}) active muscle glucose uptake can account for up to 85%. Increased skeletal muscle blood glucose utilization is due to increased glucose transport and increased activation of glycolytic and oxidative pathways that use glucose as a fuel.

The magnitude of increased skeletal muscle uptake of blood glucose is influenced by both exercise intensity and duration, and is closely tied to changes in muscle glycogen utilization (130). Although a number of mechanisms are suspected to be involved, the increased accumulation of G-6-P with increased muscle glycogen utilization appears to be the main factor controlling the relationship between muscle glycogen and glucose use due to its inhibitory effect on hexokinase.

As a result of increased uptake of blood glucose by the muscle, there is an increased release of glucose from the liver to maintain euglycemia. During exercise of low to moderate intensity, hepatic glucose output has been found to increase two- to three-fold (rest = 14.4; 41% VO_{2max} = 28; 74% VO_{2max} = 43 umol/min) (77). High intensity exercise results in far greater increases, up to seven- to ten-fold. Hepatic glucose output demonstrates a linear increase up to moderate intensity exercise (50-60% VO_{2max}), with blood glucose levels remaining fairly close to resting levels (5 mmol/l). During high intensity exercise hepatic glucose output demonstrates an exponential relationship with exercise intensity with resulting increases being in the seven-to-ten fold range. This exponential increase occurs despite a reduction in liver blood flow. As intensities increase, blood glucose levels increase, indicating that output exceeds uptake. However, research indicates that the increased level of glucose uptake by the muscle may not be matched by increased glucose metabolism, as there may be an increase in intra-muscular free glucose and G-6-P (70).

The effect of exercise intensity on blood glucose concentrations was examined by Romijn et al. (117). Five trained subjects (VO_{2max} = 67.0 ± 3.0 ml/kg/min) were studied during exercise intensities of 25%, 65% and 85% VO_{2max}. During 30 min of exercise, blood glucose concentrations increased from resting levels (70 ± 3 mg/dl) in relationship to exercise intensity (25% = 77 ± 4, 65% = 98 ± 6, 85% = 147 ± 1 mg/dl).

Two processes occur within the liver to accommodate these increased glucose needs: glycogenolysis and gluconeogenesis. The relative contribution of these two processes is determined by exercise intensity and duration. As exercise intensity is increased, there is greater reliance on glycogenolysis. As exercise duration increases, there is a shift from primarily glycogenolysis at 30 min of exercise (~90%) to a relatively even split between the two processes at 4-h (1). This shift from glycogenolysis to gluconeogenesis corresponds to a reduction in liver glycogen, an increase in hepatic fractional extraction of amino acids, and an increase in hepatic efficiency in channeling amino acids into glucose (145). Gluconeogenesis, utilizing elements from the breakdown of fats (glycerol), carbohydrates (lactate) and proteins (amino acids), is important during exercise of prolonged duration as it delays the depletion of liver and muscle glycogen.

Regulation of hepatic glucose is thought to be determined by two mechanisms: a feed-back and a feed-forward mechanism (77). Glucose infusion during moderate exercise has been shown to result in maintained levels of blood glucose concentrations with an accompanying reduction in hepatic glucose output (63). This indicates that changes in blood glucose levels feed information back to the liver, controlling levels of hepatic output. However, during more intense exercise there is a mismatch between hepatic production and glucose uptake, with blood glucose levels increasing as exercise intensity increases (50;130). These results indicate that hepatic glucose output is at least partially determined by central command and therefore, feed-forward regulation.

2.2.8 Summary of Carbohydrates

During exercise, especially as exercise intensity increases, carbohydrates are a preferred fuel in the production of energy within active muscle. During recovery from exercise, however, carbohydrates appear to be required in other ways. Hepatic glucose output is still required to maintain euglycemia for fuel for the brain and other cells, such as red blood cells, that use primarily carbohydrates as a fuel. However, within the active muscle, blood glucose and other products and intermediaries of glycolysis are required for glycogen resynthesis, perhaps reducing their rate of oxidation within active muscle. Measurement of these carbohydrate substrates both during exercise and recovery would provide a greater understanding of their availability to PDH for the purpose of oxidation within muscle and provide insight into substrate utilization, both during exercise and recovery.

2.3 Lipid Pathways and Substrates

Two steps have been identified as the most likely to be involved in the reduction and limitation of fat oxidation during increases in exercise intensity. The two steps are fatty acid mobilization from adipose tissue and transport of fatty acids into the mitochondria (65).

2.3.1 Transportation of NEFA into the Mitochondria

A recent model by Kerner and Hoppel (73) describes the multi-step process for bringing non-esterfied fatty acids (NEFA) across the mitochondrial membrane. The first step is the activation of long chain NEFA (LCFA). LCFA refer to fatty acids with a chain length between 12 and 24 carbons. LCFA must have a coenzyme-A attached before they can enter the mitochondria. The activation reaction is as follows:

$$\text{NEFA} + \text{ATP} + \text{CoA} \xrightarrow{\text{long chain acyl -CoA synthase}} \text{Fatty acyl-CoA} + \text{AMP} + \text{PPi}$$

This reaction is effectively irreversible because the product PPi is quickly hydrolyzed by inorganic pryophophatase to two Pi in a coupled reaction that drives the reaction catalyzed by long chain acyl-CoA synthase (LCAS) to the right. Activation of the fatty acid requires two phosphates (resulting in the formation of AMP rather than ADP). Medium chain fatty acids (MCFA, 8-10 carbons) and short chain fatty acids (SCFA, 6 carbons or less) pass through the two mitochondrial membranes without requiring an activation step. Following activation of LCFA, fatty-acyl-CoA passes through the mitochondrial outer membrane (MOM). Although it was generally accepted that fatty acyl-CoA passed freely through the MOM, it is now recognized that MOM is not freely permeable, and fatty acyl-CoA may cross MOM through a voltage dependent selective channel referred to as the mitochondrial porin (73).

From the intermembrane space of the mitochondria, fatty acyl-CoA is transported across the mitochondrial inner membrane (MIM) by the activity of three proteins:

carnitine palmitoyltransferase I (CPT I), carnitine:acyl-carnitine translocase (CACT), and carnitine palmitoyltransferase II (CPT II). CPT I, located within the MOM, converts fatty acyl-CoA to fatty acyl-carnitine, releasing a free coenzyme-A (CoASH) in the process. The fatty acyl-carnitine are translocated across the MIM in an exchange reaction catalyzed by CACT. Within the mitochondrial matrix, fatty acyl-carnitine is converted back to fatty acyl-CoA by the third enzyme CPT II, an enzyme anchored to the inner membrane. This process results in the release of free carnitine back into the mitochondrial matrix (73). Although numerous enzymes act upon the acyl-CoA from this point until oxidation is complete, no process past this point is considered rate-limiting in terms of fat oxidation (66).

Of the three enzymes involved in transporting FA into the mitochondria, neither CACT or CPT II are thought to play a regulatory role in mitochondrial NEFA uptake (73). CPT I is considered a key regulatory site controlling the flux of fatty acids through oxidation. CPT I is located within the MOM and, according to the model proposed by Kerner and Hoppel (73), has surface exposure to both the cytosol and the inner-membrane space. The inner-membrane surface exposure is the aspect of the enzyme where long-chain acyl-carnitine is created from long-chain acyl-CoA. The cytosolic surface contains a regulatory site which controls the activity level of the CPT I. This regulatory site is acted upon and inhibited by malonyl-CoA. It is due to this inhibition by malonyl-CoA that CPT I is considered a key regulatory site controlling the flux of fatty acids through oxidation (73). CPT I has at least two isoforms, the liver isoform (L-CPT I) and a muscle isoform (M-CPT I). M-CPT I displays a higher affinity for malonyl-CoA and a lower affinity for carnitine than L-CPT-II, making the muscle isoform more susceptible to by changes in malonyl-CoA concentrations (73).

2.3.1.1 Regulation of CPT I

CPT I is considered to be regulated by two methods: transcription and malonyl-CoA dependent regulation. Transcription appears to be induced by the accumulation of activated fatty acids, but not by their metabolism. It has been suggested that malonyl-CoA is involved in both short and long term regulation of CPT I. Acute changes in malonyl-CoA concentration result in short term regulation of CPT I. Changes in the sensitivity of CPT I to malonyl-CoA are involved in long-term regulation of CPT I (73). This thesis will focus primarily on the acute regulation of CPT I.

Recent studies have demonstrated that long-chain fatty acid oxidation is linked to changes in cytosolic concentrations of malonyl-CoA. Malonyl-CoA concentration has been demonstrated to increase in situations related to reduced levels of fat oxidation. Bavenholm et al. (10) infused high dose insulin into 16 healthy men and found malonyl-CoA significantly increased from basal rate (0.20 ± 0.01 to 0.24 ± 0.01 nmol/g) and whole-body fatty acid oxidation significantly decreased from basal rate (0.94 ± 0.06 to 0.44 ± 0.06 mg/kg/min). The increases in malonyl-CoA were associated with increases in the concentrations of citrate, an allosteric activator of Acetyl-CoA Carboxylase (ACC), and malate, an antiporter for citrate efflux from the mitochondria. Saha et al. (121) demonstrated that malonyl-CoA in mice increased in the presence of increased levels of glucose and insulin. In addition, malonyl-CoA concentration has been reported to decrease in situations of increased lipid oxidation. Winder et al. (150) demonstrated that during 120 min of treadmill running (21 m/min, 15% grade) malonyl-CoA concentrations decreased in adult male rats. Duan and Winder (31) also demonstrated that electrical stimulation resulted in decreased concentrations of malonyl-CoA. Saha et al. (120) demonstrated that malonyl-CoA concentrations in mice were decreased in muscles devoid of insulin and glucose and increased when glucose and insulin were infused.

Although research results generally support the decrease of malonyl-CoA during exercise, non-supportive results have been found. Odland et al. (98) exercised nine human subjects for 70 min (10 min at 40% VO_{2max} and 60 min at 65% VO_{2max}) and found that malonyl-CoA content did not change significantly during exercise. Odland et al. (100) also found mean malonyl-CoA levels to be similar during 10 min of exercise at three different intensities (35, 65, and 90% VO_{2max}).

2.3.1.2 Regulation of Malonyl-CoA

Malonyl-CoA formation is catalyzed by the enzyme Acetyl-CoA Carboxylase (ACC). ACC is expressed through two isoforms (ACC-α, ACC-β), with ACC-β being dominant in skeletal muscle (119). Because of its appearance in non-lipogenic muscle, it has been proposed that ACC-β is involved in the regulation of fatty acid oxidation rather than fatty acid biosynthesis (119). The unique feature of ACC-β is the first 200 amino acid residues, which are suspected to be responsible for anchoring the enzyme to the mitochondria and controlling the concentration of malonyl-CoA in the vicinity of CPT I (119).

Acute regulation of ACC takes place through covalent modification with changes in the phosphorylation state of specific serine residues by an AMP-activated protein kinase (AMPK), cAMP-dependent kinase, and/or phosphatases (119). Chronic regulation of ACC is regulated by changes in enzyme concentrations due to gene expression (119).

The acute regulation of ACC-α, more prominent in the liver, has been shown to increase during starvation (a period of increased fat oxidation) and decrease rapidly with re-feeding (a period of reduced fat oxidation). Potential regulators of ACC-α include citrate, which is both an allosteric activator and a precursor to the substrates acetyl-CoA, LCFA, and malonyl-CoA. Citrate effects ACC-α regulation using a feed-forward mechanism, while malonyl-CoA functions with a feed-back mechanism. Increases in cytosolic LCFA-CoA affect this process in two ways. The first method is by allosterically inhibiting ACC and reducing the production of malonyl-CoA. The second method is by competing with malonyl-CoA for the binding site on CTP I, and thereby diminishing the effect of malonyl-CoA (119). In addition, increased concentrations of glucose and insulin increase ACC-α activity, while glucagon and catecholamines have the opposite effect (119). ACC-β, the muscle isoform, has been shown to be allosterically activated by citrate, LCFA-CoA and malonyl-CoA, however, the effect of the other factors regulating ACC-α are not as well understood in relation to ACC-β (119).

The major protein kinase regulating ACC, acting on the last three serine residues, is 5'- AMP-activated kinase (AMPK) (119). AMPK phosphorylation of ACC results in reduced enzymatic activity and decreased sensitivity to the allosteric activator citrate. These changes are readily reversed by the activity of protein phophatases 2A and 2C (119). AMPK derives its name from the fact that it is activated by AMP (119). ACC phosphorylated in vitro by AMPK has demonstrated an increase in the Michaelis Constant (K_m) for the substrates ATP, acetyl-CoA and bicarbonate (152).

ACC is phophorylated by AMPK at three sites, two of which (Ser79 and Ser1215) seem to be specific to AMPK (45). It has been proposed that during increased physical activity AMPK not only has an effect on malonyl-CoA through ACC, but may increase the activity of malonyl-CoA decarboxylase (MCD), an enzyme that has been suggested to break down and dispose of malonyl-CoA (122). Goodwin and Taegtmeyer (41) demonstrated that when rat hearts elicited a 40% increase in lipid oxidation, there was an accompanying 33% reduction in malonyl-CoA concentration. However, it was concluded that the physiological changes in AMP were insufficient to promote ACC phosphorylation by AMPK, and that increased fat oxidation was a result of increased malonyl-CoA degradation by MCD. Recently, the relationships between ACC, malonyl-CoA and fat oxidation has been demonstrated in mice. Abu-Elheiga et al. (1) demonstrated that ACC-β deficient mice had 30-fold lower levels of malonyl-CoA in muscle and the level of fatty oxidation was 30% higher than that of mice not ACC-β deficient.

It has been proposed that AMPK, through its eventual effect on malonyl-CoA, is involved in the switch in principal fuel utilization from carbohydrate to fat which occurs during prolonged exercise (122). It is futher proposed that AMPK represents a protein kinase cascade that provides a sensitive system acting as a cellular fuel gauge within the cell. Under resting conditions, a high ATP/AMP would result in the dephosphorylation and inactivation of AMPK. This would result in ACC being in its active form, with a resulting increase in malonyl-CoA production and resulting inhibition of CPT I and absolute fatty acid oxidation (73). At the onset of exercise, ATP/AMP would decrease, resulting in activation of AMPK, deactivation of ACC, reduction of malonyl-CoA concentration and increases in absolute fatty acid oxidation.

This hypothesized role of AMPK in fat metabolism has been supported by research investigating the effect of chronic activation of AMPK on resting rat muscle (153). Chronic activation resulted in significant increases in activation of numerous enzymes of the TCA cycle and the electron transport chain, particularly in white muscle. Hexokinase and GLUT-4 were increased in both red and white fast quadriceps. In addition, there was a marked decrease in the fat pad size. Winder et al.(153) stated that these results indicate that activation of AMPK may play a role in mediating some of the biochemical adaptations that are induced in skeletal muscle by endurance exercise and suggest some rationale for the role of AMPK activators to treat obesity (153).

2.3.1.3 AMPK Structure

AMPK is a hetero-trimer composed of one catalytic subunit (α) and two regulatory subunits (β and γ). Isoforms of all three subunits have been identified. Expression of the different isoforms varies dramatically between tissues and leads to the speculation that they play a role in tissue-specific function and/or regulation of AMPK activity (24).

Skeletal muscle has been reported to contain higher expression of the $\alpha2$ and $\beta2$ and γ 1 isoforms (24). In muscle, $\alpha2$ complexes have been demonstrated to account for approximately 80% of total AMPK activity with $\alpha1$ accounting for the remaining 20% (24). Fujii et al. (37), in exercising subjects at 50 and 70% VO_{2max}, concluded that the AMPK complex containing $\alpha2$ rather than $\alpha1$, may be involved in metabolic responses to exercise. Musi et al. (92) found that exercise at 70% VO_{2max} for 20-min significantly increased AMPK $\alpha2$ activity 2.7-fold over basal, but that there was no effect on AMPK $\alpha1$ activity. Wojtaszewski et al. (155) reported that $\alpha2$ AMPK, isolated from human skeletal muscle, displayed a greater dependency on AMP *in vitro* than did α1. Wojtaszewski et al. speculated that these results indicate that α1-containing AMPK complexes do not play an important physiological role in the regulation of metabolism in human skeletal muscle during exercise. However, Chen et al.(22), using a higher intensity of exercise (maximal cycle exercise for 30 s), found a similar increase (~ two-fold) in AMPK α1 and α2 activity (measured in the presence of AMP). Therefore, α1 activation may require higher exercise intensities than α2. It has also been speculated that the differences in response of α1 and α2 are a result of having different locations within the cell (155).

2.3.1.4 AMPK Regulation

AMPK is activated by AMP and AMPK kinase (AMPKK). AMPKK is an upstream kinase that results in phosphorylation and activation of AMPK. AMPKK is activated by AMP. Hardie and Carling (45) have developed a hypothesis for AMPK activation by AMP and AMPKK. The effect of AMP on AMPK is two-fold: covalent (phosphorylation through activation of AMPKK) and allosteric activation. This results in AMP having four different effects on AMPK: direct allosteric activation of AMPK, binding to dephosphorylated AMPK (making it a better substrate for AMPKK), binding to phosphorylated AMPK, and direct activation of AMPKK. Hardie and Carling speculated that AMPK regulation exists in four unique states. Two are conformational states: a less active state (T) and a more active state (R). Two other states are phosphorylated states: phosphorylated and dephosphorylated. Attachment of AMP to AMPK results in a transformation from T to a stabilized R state. Phophorylation of AMPK by AMPKK occurs most significantly when AMP is in the R state. It is proposed that attachment of AMP to AMPK exposes the phosphorylation site (Thr172) to the upstream kinase, AMPKK. Conversely, detachment of AMP from AMPK results in the covering of the phosphorylation site, causing reduced phosphorylation by AMPKK (45). It has been reported that there is substantial AMPKK activity at rest in humans (23), in marked contrast to low resting levels of AMPK. This indicates that there must be other factors, perhaps cellular AMP levels, that are important to maintaining AMPK in its inactive dephosphorylated state at rest (23).

Both roles of AMP affect the activation level of AMPK. The allosteric activation of AMPK by AMP increases its activity approximately five-fold. The activation of AMPKK by AMP and its subsequent phosphorylation of AMPK, result in an at least 50-fold increase in AMPK activity. Present research is unclear as to whether the dephosphorylated forms exhibit any level of AMPK activity (45). With this model it is believed that most stages are antagonized by high concentrations of ATP, and it is believed that the signal to which AMPK responds is more affected by the AMP/ATP ratio than simply the concentration of AMP.

The dual role of AMP in activating AMPK makes it a key element in the chain resulting in increased fat oxidation. There is only a relatively moderate increase in muscle AMP concentration in rodent skeletal muscle during exercise due to the rapid conversion of AMP to inosine monophosphate (IMP). However, AMPK activity is readily increased, presumably as a result of increased free [AMP] (22). Howlett et al. (59) investigated free AMP levels at rest and during 10-min of exercise at 35%, 65% and 90% VO_{2max} with seven untrained human subjects. Results showed that AMP levels were unchanged at any time during exercise at 35% or 65% VO_{2max}. At 90% VO_{2max} AMP was greater than both

35% and 65% at 1 and 10 min. In addition, AMP levels during 90% VO_{2max} were significantly greater at 10 min than 1 min, indicating a gradual increase in AMP during exercise at 90% VO_{2max}. Chen et al. (22) measured free AMP levels before and after a maximal 30 s sprint. Free AMP increased from rest to end exercise (rest: 0.37 ± 0.08; 30-s: 1.11 ± 0.33 *u*mol/kg dry muscle). In addition, the ratio of AMP/ATP increased significantly from rest to end-exercise (rest: 0.016 ± 0.003; 30-s: 0.051 ± 0.016 *u*mol/kg dry muscle). These results indicate that AMP accumulation is affected by exercise intensity.

2.3.1.5.AMPK Measurement

The AMPK assay utilized in this thesis measures the incorporation of ^{32}P-labelled phosphate from [^{32}P]ATP into an artificial peptide substrate resembling an AMPK phosphorylation target site on acetyl-CoA carboxylase (ACC). An increase in AMPK activity in this assay represents increases in activation due only to the phosphorylation of AMPK. AMP can allosterically activate AMPK and creatine phosphate (PCr) can allosterically inhibit AMPK, however both AMP and PCr are lost in one of the supernatants discarded in the assay process (149).

2.3.2 AMPK and Fat Oxidation

The muscle isoform of ACC is activated by citrate and the level of activation of the enzyme is dependent upon its phosphorylation state (141;151). Research utilizing animals has demonstrated a concurrent activation of AMPK and inactivation (phosphorylation) of ACC due to electrical stimulation (61) or exercise (113;151). Five-amino-4-imidazolecarboxamide riboside (AICAR) has been used as an activator of AMPK. After entering the cell, AICAR is metabolized to its monophosphorylated form ZMP and increases the activity of AMPK by mimicking the effects of AMP (52). Utilization of AICAR to activate AMPK in rats has resulted in increased phosphorylation of ACC, reduced malonyl-CoA and increased fatty oxidation rates (86;87). However, it is important to note that AICAR injections do not mimic precisely the high-energy phosphate changes that occur during contraction (149).

The role of AMPK in controlling the enzymatic cascade from ACC to CPT1 and increased fatty acid oxidation is less well defined in human studies (149). Most human studies (22;29;136), but not all (157), have demonstrated reduced ACC activity with exercise. Stephens et al. (136) had seven individuals cycle for 30 min at 63% VO_{2max} and acquired muscle biopsies at 5- and 30-min of exercise and reported AMPKα2 activity and ACCβ phosphorylation increased progressively during exercise. Dean et al. (29) had thirteen males perform one-legged knee-extensor exercise at 60, 85 and 100% of knee extensor VO_{2max} and reported a 50-75% diminishing of ACCβ activity generally in proportion to exercise intensity. In contrast, when six males cycled until exhaustion (~3.5 h) there was a gradual increase in AMPK activity, while the phosphorylation of ACCβ peaked at 1-h of exercise and had returned to resting levels at exhaustion. This led the authors to suggest that marked activation of AMPK was not sufficient to maintain an elevated ACCβ phosphorylation during prolonged exercise (157). In addition, exercise has resulted in a small decline in malonyl-CoA in one group (29) but not in others (98;99). Speculative reasons for the more equivocal results with humans may be that malonyl-CoA concentrations are much lower in human muscle than in rat muscle, particularly in the relatively small human muscle biopsies. In addition, it is an easier process to obtain samples of homogenous fiber type in rats than humans. Another possibility is that ACC in human muscle is arranged in close proximity to CPT-1. Therefore, total malonyl-CoA may not be as representative of the effect of ACC activity as the concentration localized around CPT-1 (149).

Exercise that produces the greatest level of AMPK activation should then bring about the greatest increases in fatty-acid oxidation. Research has demonstrated increased AMPK activity during exercise with increased workload (23;37;155). However, research on fat oxidation generally tends to follow a different pattern. Fat oxidation tends to peak during moderate exercise, with further increases in intensity resulting in reduced absolute and relative fat oxidation (23;117;139).

Considerable research has been performed in an attempt to describe the regulatory mechanisms that may occur in the reciprocal relationship between carbohydrate and fat utilization during increases in exercise intensity. In a review of the interaction between carbohydrate and fat during exercise, Spriet et al. (131) suggested two theories as to why fat oxidation would be inhibited as exercise intensity is increased. Increased carbohydrate oxidation, which occurs as exercise intensity increases, results in increased cytosolic citrate and acetyl-CoA. These two metabolites both stimulate ACCβ activity, which would result in increased malonyl-CoA, inhibited CPT1 and reduced fat oxidation. However, muscle malonyl-CoA concentrations have not been found to increase in rat or human studies during high intensity exercise (100;113), therefore this mechanism is unlikely to lead to reduced fat oxidation (139). Secondly, Spriet et al. proposed that decreases in muscle pH, which occur at moderate and intense exercise outputs (59), induce large reductions in CPT1 activity with resulting reductions in fat oxidation. A third possible mechanism was proposed by van Loon et al. (139) involving the availability of muscle carnitine. Carnitine is a co-factor required for transporting long-chain fatty acids across the inner mitochondrial membrane, a function essential for fat oxidation to occur (18). However, as exercise intensity and PDH activity increase, the rate of production of acetyl-CoA exceeds its rate of utilization in the tricarboxylic acid (TCA) cycle. Under these conditions carnitine acts as a sink for acetyl group storage forming acetyl-carnitine. This action reduces the availability of free carnitine for fat oxidation and fat oxidation is therefore reduced. Eight subjects were cycled for 30 min at 40, 55 and 75% of maximal workload and the authors concluded that the most likely mechanisms for the reduction of fat oxidation during high intensity exercise were a marked decline in free carnitine or a decrease in intracellular pH (139).

2.3.3 AMPK and Glucose Uptake

Recent research suggests a positive link between increased AMPK activity and increased glucose uptake (2;56;101), with this increase being additive to those increases due to the effect of insulin (149). Holmes et al. (56) injected rats with AICAR for 5 days and reported that GLUT-4 increased between 60-100% in skeletal muscle, a similar increase to that which would have occurred with endurance training. *In vitro* incubation of rat epitochlearis muscle in AICAR containing culture for 18-h also produced a ~50% increase in GLUT-4 (101). Studies using electrical stimulation of incubated rat muscle have also demonstrated a positive relationship. Ai et al. (2) stimulated rat muscle at four different frequencies and found that muscle glucose transport increased with stimulation frequency and increased AMPK activity. Jessen et al. (64) allocated rats into three groups: an exercise group, an AICAR exposed group, and a sedentary control group. The exercise and AICAR group both displayed fiber-type-specific (greatest increase in IIb fibers) increases in glucose transport with a concomitant increase in GLUT-4 expression.

Recent studies have utilized genetically altered animal muscle to investigate this area. Sakoda et al. (123) utilized rat skeletal muscle altered for dominant negative mutant AMPKα2 and found that rats with reduced AMPK activity (dominant negative mutant) produced a reduced effect on AICAR-induced glucose uptake, supporting the role of AMPK in glucose uptake.

Some investigations are questioning the role of AMPK in this area. Wright et al. (158) tested the different roles of AMPK and calcium in glucose uptake. Caffeine was used to increase calcium to a level too low to cause contraction. In addition, AICAR was used to activate AMPK. The results demonstrated that the increases in glucose transport induced by AICAR and caffeine were additive, leading to the conclusion that both calcium and AMPK are involved in the stimulation of glucose transport by muscle contractions. In addition, in further research with kinase-inactive AMPK mutant mice, AICAR-stimulated glucose uptake was completely abolished, whereas contraction-stimulated glucose uptake was only partially reduced (89). Collectively, these results would tend to show that while AMPK plays a role in the process of glucose uptake, it does not appear to be indispensable and other mechanisms seem to be involved (93).

2.3.4 AMPK and Glycogen Concentration

Recent investigations in animals and humans indicate that glycogen content may modulate the AMPK response to contraction (2;30;71;136;156). Derave et al. (30) utilized rats with high and low muscle glycogen levels. Electrical stimulation resulted in greater AMPK activation in the low muscle glycogen rats in both red and white skeletal muscle. Ai et al. (2) electronically stimulated incubated rat muscle at four different frequencies and found that AMPK activity increased with increased stimulation frequency and reduced muscle glycogen concentration. Recently, increased levels of muscle glycogen has been demonstrated to significantly blunt the AICAR and contraction stimulated increase in AMPK activity in rats (71;154).

In humans, eight athletes cycled for 1-h of exercise at 70% peak oxygen consumption in a glycogen-loaded or glycogen-depleted condition. During exercise AMPKα2 activity was higher in the glycogen-depleted condition. This occurred without any apparent differences in muscle high-energy phosphates (156). Stephens et al. (136) cycled seven active healthy individuals for 30 min at 63% VO_{2max} and reported that increases in AMPKα2 activity were more closely coupled with reductions in muscle glycogen than with the muscle AMP/ATP ratio. Whether glycogen concentration has a direct effect on AMPK activity, or the reduced activation is a result of the blunting of changes in ATP, AMP and PCr is unclear (149). In short, the presence of high glycogen in muscle appears to attenuate the increase in AMPK during exercise (149). No human research has compared AMPK activation with exercise protocols that would produce significantly different reductions in muscle glycogen.

2.3.5 AMPK during Exercise and Recovery

In humans, cycling exercise has been demonstrated to increase AMPK activation in a positive relationship to intensity (23;37;155) and duration (157). Wojtaszewski et al. (155) had seven healthy men cycle for 90-min at 50% VO_{2max} and 60 min at 75% VO_{2max} and found an increase in AMPKα2 activity only from the higher intensity treatment. Chen et al. (23) had eight men cycle for 20-min at approximately 40, 60 and 80% VO_{2max}

and reported a significant increase in AMPKα2 activity as intensity increased. Only one investigation has looked at interval style exercise. Trained cyclists and untrained men performed repeated intervals of five-min at 85% VO_{2max} and demonstrated increases in AMPKα2 activity of 4.2 fold and 2.3 fold in untrained and trained respectively (159).

Three studies have investigated AMPK activity during recovery from exercise. Fujii et al. (37) measured AMPK activity 30 minutes into recovery for seven subjects (4 males, 3 females) following cycle exercise at 70% VO_{2max} and found increased AMPKα2 activity. However, the other two investigations, using exercise treatments that increased AMPK activity, found no increase in recovery AMPK activity when measured at 1-h (157) and 3-h post-exercise (155).

Only one investigation has compared two different exercise treatments that were matched for work, and that investigation dealt with relatively moderate exercise intensities (155). An investigation utilizing moderate exercise and high intensity interval exercise matched for work and duration would assist in understanding the effect of exercise intensity on AMPK activity.

2.3.6 AMPK and Endurance Training

In an attempt to understand the effect of endurance training on AMPK activity, Frøsig et al. (36) had eight healthy young men perform supervised one-legged knee extensor endurance training for 3 weeks with biopsies obtained before training started and 15 hours after the last training sessions. AMPK resting activity increased in the trained leg and phosphorylation of ACCβ increased by 180%, despite increased glycogen content in the trained leg, leading the authors to conclude that basal AMPK activity in human skeletal muscle is highly susceptible to endurance exercise training. Nielsen et al.

(96) investigated the activation of AMPK in trained and untrained human muscle in seven endurance exercise-trained and seven sedentary young healthy men. Basal AMPKα1 activity was 185% greater in trained than untrained, with all other subunits being similar. After 20-min of exercise at 80% VO_{2peak}, the increase in phosphorylation of AMPKα2 was blunted in the trained group (138% above rest) compared with the sedentary group (353% above rest). In addition, ACCβ phosphorylation was increased by exercise in both groups but to a lower level in trained than sedentary. The author stated that these results may help explain the role of AMPK in performance improvement, in that with a long term training program a continued progressive improvement of exercise performance and training-induced cellular adaptations are generally dependent upon a progressive increase in training volume and intensity. Therefore, they speculate that the blunted AMPK activity in trained subjects working at the same relative intensity as sedentary subjects may in part explain this phenomenon. Reduced AMPK activity despite higher absolute energy requirements in the trained subjects may be a result of better maintenance of the cellular energy charge as reflected by higher PCr/Cr ratios observed in the trained subjects during exercise. In addition, the authors state that these results demonstrate the important point that AMPK activation is not a marker of total energy flux during exercise but rather a result of the perturbations in the energy charge induced by exercise (96).

2.3.7 AMPK and Gene Expression

Chronic activation of AMPK, as reported earlier, has been demonstrated to result in increased proteins related to glucose metabolism in skeletal muscle, including GLUT-4 and hexokinase (56;101). Recent research has investigated the role of AMPK in the adaptations that occur in mitochondrial proteins as a result of repeated bouts of exercise. Putman et al. (106) infused rats with AICAR for 28 days and reported significant increases in uncoupling protein-3 (UCP-3) as well as increased activity of the mitochondrial enzymes citrate synthase and 3-hydroxyacyl-CoA-dehydrogenase, two enzymes that are known to increase in proportion with mitochondrial volume density. Winder et al. (153) chronically activated rats for 28 days with AICAR and reported similar increases in mitochondrial enzymes in white but not red quadriceps muscle, with the authors stating that these results indicated that chronic AMPK activation may mediate some of the biochemical adaptations to muscle that occur during endurance exercise training. McGee et al. (85) had six males cycle for 60 min at ~72% VO_{2peak} and nuclear AMPKα2 content was approximately doubled. As there was no change in whole-cell AMPKα2 content, it was suggested that nuclear translocation of AMPK might mediate the effects of exercise on skeletal muscle gene and protein expression (85). These results suggest that the activation of AMPK is involved in some of the biochemical adaptations and gene transcriptions that take place during and as a result of exercise training (93).

2.3.8 AMPK and Clinical Considerations

When the varied actions of AMPK are considered, such as activation of glucose transport, GLUT-4 translocation, fatty acid oxidation and increased gene expression of mitochondrial proteins, it is not difficult to imagine that a deficiency in AMPK could lead to obesity. Logically then, pharmaceutical activators of AMPK may provide a novel approach for the treatment of obesity (149).

AMPK activators, such as AICAR, metformin (91) and leptin (88), provide a method of chronically activating AMPK to bring about these positive results. Exercise, however, is likely the safest method of chronically activating AMPK. Schrauwen et al. (124) demonstrated that a 12-week training program of low-to-moderate intensity (40% VO_{2max}) resulted in reduced ACCβ mRNA expression and greater fat oxidation. In an investigation of trained vs. untrained subjects, it was demonstrated that trained subjects had increased AMPK protein levels (96). In addition, three weeks of one-legged knee extensor endurance training demonstrated increased basal AMPK activity and increased protein expression of both catalytic and regulatory subunits of AMPK in the trained over the untrained limb (36). The effect of this training appeared to persist 55-h after the last bout of exercise leading the authors to conclude that AMPK activity is chronically increased during periods of regular repeated exercise. A greater understanding of what exercise protocol most effectively activates AMPK during both exercise and recovery may prove valuable in understanding how to maximize the many and varied benefits related to AMPK activation.

2.3.9 Lipid Substrates

A number of recent reviews have examined the role of lipids during exercise and recovery (55;65-67;83;95;110;114). The primary storage area for fats is adipose tissue. Adipose tissue is located in two primary areas: below the skin (subcutaneous) or deeper within the body (visceral). A secondary storage area for fats is within muscles and is referred to as intramuscular triglycerides (IMTG). The size of the adipose fat storage is considerably greater than IMTG. Calculated lipid energy stores of an 80 kg man estimate there would be 12 kg of adipose tissue, with a resulting energy capacity of 404,000 kJ, and an IMTG store of approximately 0.3 kg (range 0.1-0.6) for an energy capacity of 10,800 kJ. The storage of IMTG is generally in the range of 7 to 40 mmol/kg/wet wt.

Type I fibers have been shown to have higher concentrations of IMTG than Type IIa, with IMTG being at very low concentrations in IIb fibers.

Fatty acids not esterfied to mono-, di- or triglyceride will be referred to in this thesis as non-esterfied fatty acids (NEFA). NEFA contain even number carbon chains which vary in length from less than six carbons to 24 carbons. There are three categories of NEFA related to carbon chain length. Long chain NEFA (LCFA) are of C12-C24 in length, with the most common long chain fatty acids in the typical North American diet being palmitic (C16) and oleic acids (C18). Medium chain NEFA (MCFA) are C8-C10 in length. Fatty acids containing six carbons or less are referred to as short chain NEFA (SCFA).

Oxidizable lipids include NEFA, circulating triacylglycerols (CTG) and intramuscular triglycerides (IMTG). NEFA and intramuscular triglycerides constitute the vast majority of fats oxidized during exercise. CTG include chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL) and high density lipoproteins (HDL). The primary difference in these forms of CTG is their triglyceride and cholesterol content and their function. Chylomicrons and VLDL may play a role in energy metabolism, whereas the other lipoproteins likely do not. VLDL and LDL are the main lipoprotein species for the transport of triglycerides from the liver to adipose tissue and muscle. HDL primarily transport cholesterol from peripheral tissue to the liver.

2.3.9.1 NEFA during Exercise and Recovery

Two steps are considered most likely in the limitation of fat oxidation. The first step is the transport of fatty acids into the mitochondria. The second step is the mobilization of fatty acids from the adipose tissue. The rate of mobilization from adipose tissue is dependent upon the following factors: rate of lipolysis, rate of reesterification of NEFA within the adipocyte, and the transport of NEFA from adipose tissue in blood (65). Lipolysis in the adipocyte is initiated with the breakdown of adipocyte triglycerides by the enzyme hormone sensitive lipase (HSL). HSL can be activated by an adrenergic stimulated cascade. Action of HSL, the rate-limiting step in mobilization, upon triglycerides results in the formation of a monoacylglycerol and two NEFA. The monoacylglycerol is then broken down by the enzyme monoacylglycerol lipase (MGL) into glycerol and another NEFA. The resulting three NEFA and one glycerol then diffuse into the circulation (65).

A number of factors contribute to enhancing lipolysis, including catecholamines, glucocorticoids, thyroid stimulating hormone and growth hormone. In contrast, other factors inhibit adipocyte lipolysis, including ketone bodies, lactate and insulin (65). During exercise plasma catecholamines and sympathetic neural activity rise exponentially with increasing exercise intensity (66). Growth hormone also increases in proportion to exercise intensity and has been shown to have a facilitating effect on catecholamines (66). Although insulin concentration is decreased with exercise, lactate concentrations have been shown to increase in proportion to exercise intensity (66). These factors are involved in controlling the rate of lipolysis in adipose tissue.

The rate of adipose tissue lipolysis is generally monitored by measuring the rate of glycerol release from adipose tissue. Glycerol cannot be reused for the esterfication of new triglycerides (TG) within the adipocyte due to the small concentrations of, or total lack of, the required enzyme glycerol kinase. Therefore, all glycerol produced by adipose lipolysis is released into plasma. Because of this, the appearance of glycerol in plasma is used as a measurement of whole-body lipolysis. With complete hydrolysis of adipose tissue TG, the release of 1 molecule of glycerol will be accompanied by the release of 3 molecules of NEFA. However, during rest, the release ratio of NEFA/glycerol is usually between 1-2 with a portion of the fatty acids remaining within the adipocyte and undergoing re-esterfication. NEFA re-esterfication to form new TG can occur within the adipocyte or within other organs of the body (liver) (65). The process of reesterfication is often referred to as triacylglycerol-fatty acid cycling (5).

The rate of NEFA removal from adipose tissue is often quite different from glycerol release and is dependent upon plasma albumin concentration, the arterial NEFA /albumin (NEFA /A) ratio, and blood flow through the adipose tissue. NEFA bind with albumin with decreased affinity, resulting in increases in free NEFA (non-albumin bound NEFA in plasma) as the NEFA/A increases. The plasma albumin concentration remains fairly stable (approximately 0.6 mmol/l). The plasma fatty acid concentration however, is more variable. In most conditions, fatty acid concentrations vary between 0.2 – 1.0 mmol/l. However, during moderate intensity exercise, arterial concentrations may increase up to twenty-fold. This increase results in a change in the NEFA /albumin ratio from a resting value of 0.2 to a value of 3-4 during exercise (65).

NEFA released from the adipocyte have two fates: metabolism within the various fat burning cells of the body, or reesterfication. During periods of low blood glucose, the uptake of glucose and its conversion into Glycerol-3-Phosphate (G-3-P) is decreased. G-3-P is required to bind to acyl-CoA in the synthesis of TG in liver or adipose tissue. Therefore, the reduction in blood glucose results in a reduced rate of reesterfication, resulting in greater numbers of NEFA available in the blood for metabolism. High intensity exercise has been shown to result in greater depletion of both muscle and liver glycogen than lower intensity activity. It is possible that the increased fat utilization following higher intensity exercise may be partially in response to greater amounts of NEFA and reduced levels of blood glucose. Measurement of NEFA and blood glucose during the recovery from high intensity interval and moderate intensity continuous exercise may provide greater insight into the mechanisms involved in the post-exercise substrate shift towards greater fat metabolism shown after higher intensity exercise (103).

Mulla et al. (90) looked at substrate availability at two different moderate intensities (40 and 60% VO_{2max}) and found no difference in glycerol or NEFA concentrations, pre-exercise, during exercise or during a 3-h post-exercise period. Their results demonstrated that glycerol and NEFA increased over exercise duration (60-90 min) at moderate exercise intensities. Similar to Romijn et al. (117), Mulla et al. (90) found that glycerol concentration decreased and NEFA increased in the first 10-min post-exercise at both intensities.

Romijn et al. (117) investigated five trained subjects during and after exercise at three different intensities (25, 65 and 85% VO_{2max}) using a stable isotope tracer method and indirect calorimetry methodologies. Plasma glucose response was found to be closely related to intensity, as the rate of glucose appearance (Ra), plasma glucose concentration and the rate of glucose disappearance (Rd) were all increased in relation to exercise intensity. Plasma glucose concentrations were 77 ± 4, 98 ± 6 and 147 ± 1 mg/dl after 30 min of exercise at 25, 65 and 85% VO_{2max} respectively above pre-exercise levels (70 ± 3 mg/dl) (117).

In contrast, peripheral lipolysis was stimulated maximally at the lowest exercise intensity and fatty acid release into plasma decreased with increasing exercise intensity (117). NEFA concentrations steadily increased during exercise at 25% VO_{2max} from a pre-exercise level of 0.86 ± 0.12 mmol/l to a 30 min level of 0.97 ± 0.18 mmol/l. During exercise at 65% VO_{2max}, NEFA concentration decreased from pre-exercise levels (0.86 ± 0.14 mmol/l) in the initial five minutes (0.67 ± 0.13 mmol/l) but then steadily increased to the 30-min mark (0.90 ± 0.14 mmol/l). During exercise at 85% VO_{2max}, NEFA concentrations decreased from resting values (0.99 ± 0.05 mmol/l) to the 30 min mark (0.52 ± 0.08 mmol/l)(117). In contrast to the relationship between NEFA concentrations and exercise intensity, glycerol concentrations increased during exercise of all intensities (117). Post-exercise, the relationship between glycerol and NEFA concentrations was also inverted. Immediately post-exercise, Romijn et al. demonstrated an increase in plasma NEFA concentration. This increase was positively related to exercise intensity, with the greatest increases occurring after the highest intensity exercise (85% VO_{2max}). Glycerol concentration, however, steadily declined to resting levels after all exercise protocols.

Measurement of whole body fat oxidation rates during exercise supported these results. Fat oxidation after 30 min was not different between 25% and 85% VO_{2max} in spite of the more than three times greater energy requirement. Fat oxidation at 65% VO_{2max} was significantly greater than at 25% VO_{2max}. These results provide a relationship between fat oxidation and exercise intensity. During low intensity exercise, peripheral lipolysis was high and fat metabolism was accounted for almost entirely by NEFA. Whole body fat oxidation was highest during moderate intensity exercise, however, there was a slight reduction in NEFA oxidation, thus indicating a significant contribution from intra-muscular triglycerides (IMTG). At high intensity, whole body fat oxidation was similar to low-intensity. However, during low intensity exercise, NEFA were the dominant lipid fuel utilized. During high intensity exercise, fuel for fat oxidation was contributed equally by NEFA and IMTG. Romijn et al. performed this protocol utilizing men (117) and women (116) and found similar results, indicating similar substrate utilization between men and women under these circumstances.

This study is the most widely referenced article on the issue of substrate utilization during and after exercise of different intensities. However, the level of fitness of the subjects in this study (men: VO_{2max} 67 ± 3 ml/kg/min, women: VO_{2max} 70 ± 2 ml/kg/min) was high and evident in their ability to maintain an exercise intensity of 85% VO_{2max} for 30 min. No investigations could be found that examined blood-borne substrates during interval exercise. In addition, no studies could be found that compared high intensity exercise to moderate exercise of a duration and magnitude that could be performed by recreational athletes and people using exercise as a weight loss tool. There is also a lack of research examining blood borne substrate utilization during recovery from interval exercise. Measurement of these substrates during recovery could provide greater understanding of the mechanisms involved in increased fat oxidation following high intensity exercise.

2.3.9.2 IMTG during Exercise and Recovery

The role of IMTG during exercise is covered by a number of recent reviews. (47;65;66;83;110;127;146). The storage of IMTG within human muscle cells is generally in the form of lipid droplets within the cytoplasm in close proximity to the mitochondria

(12). Storage by fiber type is generally positively related to oxidative capacity, with Type I fibers typically containing greater amounts of IMTG than Type IIa fibers, and type IIb fibers displaying the lowest concentrations. Training appears to increase IMTG stores within all muscle fibers.

Knowledge pertaining to the exact mechanisms of IMTG metabolism within the muscle fiber is very limited (65). Evidence appears to indicate that a neutral triglyceride lipase (TGL), a form of lipoprotein lipase, is responsible for the breakdown of IMTG. This neutral TGL is likely under hormonal control and influenced by the adrenergic system. Decreases in the concentrations of IMTG have been brought about by infusion of norepinephrine and stimulation of β-adrenergic receptors. Cleroux et al. (26) attempted to determine the specific β-adrenergic isoform responsible for IMTG lipolysis. IMTG utilization was blocked by the use of a non-selective β-blocker, however, IMTG was unaffected when a β1-adrenergic blocker was utilized. Other investigations have demonstrated that IMTG utilization is increased in slow-twitch fibers, which have higher densities of β2-adrenoceptors. These results seem to indicate that IMTG lipolysis is partially mediated through β2-adrenergic stimulation. It has also been speculated that increased [Ca^{2+}] plays a role (65).

Studies that have investigated the use of IMTG during exercise have recently gained greater attention as a result of the proposed relationship between increased IMTG stores and metabolic disorders such as insulin resistance (11), but results are equivocal (65). Studies using ^{14}C-labelled NEFA to estimate IMTG utilization have generally speculated that IMTG utilization is significant during exercise (44). These studies determine that total lipid oxidation, as calculated from the respiratory exchange ratio

(RER), is greater than NEFA oxidation. This difference is speculated to be the contribution of IMTG to metabolism (47). Romijn et al. (117) predicted that IMTG utilization is relatively insignificant at lower intensities (25% VO_{2max}), and reaches its peak at moderate intensities (65% VO_{2max}). At higher intensities, IMTG utilization is believed to decrease, due primarily to a reduced ability of the mitochondria to uptake intracellular NEFA (66). The drawback to these investigations is the fact that IMTG utilization is estimated, and not directly measured.

Investigations that attempted direct measurement of IMTG utilization by the muscle biopsy technique have also demonstrated equivocal results. Some studies have found that IMTG decreases from exercise (17;32;35) while others have not (62). IMTG concentration in vastus lateralis muscle has been demonstrated to decrease from 25-50% during prolonged exercise at moderate exercise intensity (55-75% VO_{2max}) (47). Continuous high intensity exercise of 5 min of cycling has decreased IMTG concentration by 29% (47). However, no change in IMTG was reported after intermittent stimulation at high intensity (75), suggesting that a portion of IMTG is constantly being resyntheisized and hydrolyzed during the lower intensities or rest phases of interval or intermittent exercise (47).

Some problems that have been identified with the muscle biopsy technique are that the extracted biopsy represents only a small part of the whole muscle and might not be representative of whole muscle IMTG stores. IMTG stores are distributed more heterogenously over the muscle compartments than are the more homogenous stores of muscle glycogen. In addition, biopsied muscle cells are often surrounded by extracellular fat. This fat, although not speculated to contribute significantly to exercise or post-exercise metabolism, must be dissected before IMTG analysis (47). Due to these difficulties, it has been estimated that only changes in IMTG larger than 24% may be detectable (147).

Information on IMTG utilization during recovery is limited. Eight subjects were fed carbohydrate-rich diets and then performed cycle exercise to exhaustion with no significant muscle glycogen reduction (75). However, IMTG concentrations, measured with the biopsy technique, decreased during the first 18 h after exercise, leading the authors to suggest that IMTG provide an important fuel for muscle metabolism during the recovery period when muscle glycogen resynthesis has high metabolic priority. Two recent investigations contradict this result. Eight trained male subjects performed 120 min of moderate intensity exercise and 120 min of post-exercise recovery. Fluorescence microscopy techniques revealed IMTG utilization during exercise but no subsequent change during recovery (140). In support, no net IMTG utilization during recovery was demonstrated throught the biopsy technique with endurance trained males performing exhaustive exercise (~90 min) (76). In summary, the utilization of IMTG during exercise and recovery is still controversial, and more research is required to clarify the its role.

2.4.1 Summary of Exercise and Recovery Substrate Utilization

Past research (78;84) has reported significantly higher respiratory exchange ratio (RER) values during, and lower RER values following, high intensity interval exercise compared to moderate intensity continuous exercise of equal work. However, some of the underlying metabolic controls responsible for regulating carbohydrate and fatty acid oxidation during exercise and recovery are not fully understood. Pyruvate dehydrogenase (PDH) controls the flux of pyruvate-derived acetyl-CoA into the Krebs cycle, and thus controls the rate of terminal glucose oxidation (108). During exercise, PDH activity is positively related to exercise intensity (59), yet during recovery it has only been studied in humans simultaneous with carbohydrate feeding (76). The 5'-AMP-activated protein kinase (AMPK) is an important positive regulator of fatty acid oxidation within muscle cells (72;152). However, AMPK activation during exercise using treatments that differ in intensity but are equal in total work and duration has not been investigated. In addition, the recovery period from these two exercise protocols warrants further investigation in light of the different RER values observed in past investigations (78;103). Research examining the effect of interval and continuous exercise of equal work and duration on PDH$_a$ and AMPK activation, along with patterns of substrate utilization and availability during the post-exercise recovery period may provide beneficial information regarding exercise prescription for performance enhancement (96), and for treating metabolic disorders that underlie obesity and type 2 diabetes (118).

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CHAPTER 3

EXCESS POST-EXERCISE OXYGEN CONSUMPTION FOLLOWING CONTINUOUS AND INTERVAL CYCLING EXERCISE

(a version of this manuscript has been accepted for publication in International Journal of Sport Nutrition and Exercise Metabolism)

3.1 Abstract

The purpose of this investigation was to examine the effect of interval (INT) and continuous (CON) cycle exercise on excess post-exercise oxygen consumption (EPOC). Twelve males first completed a graded exercise test for VO_{2max} and then the two exercise challenges in random order on separate days approximately one week apart. The INT challenge consisted of seven 2 min work intervals at 90% VO_{2max}, each followed by 3 min of relief at 30% VO_{2max}. The CON exercise consisted of 30-32 min of continuous cycling at 65% VO_{2max}. Gas exchange and heart rate (HR) were measured for 30 min before, during and for 2 h post-exercise. Three methods were used to analyze post-exercise oxygen consumption and all produced similar results. There were no significant differences in either the magnitude or duration of EPOC between the CON and INT protocols. However, HR was higher ($p<0.05$) while respiratory exchange ratio (RER) was lower ($p<0.05$) following INT. These results indicate that when total work was similar, the magnitude and duration of EPOC are similar following CON or INT exercise. The differences in HR and RER during recovery suggest different physiological responses to the exercise challenges.

3.2 Introduction

A number of authors have stated the importance of excess post-exercise oxygen consumption (EPOC) to energy balance and weight management (1;3;7;8;14-16). Sedlock et al. (16) reported that the energy value of EPOC was equivalent to approximately 10% of the exercise energy expenditure following 300 kcal of exercise at 75% VO_{2max}. Bahr et al. (3) reported an EPOC of approximately 15% of exercise energy expenditure following 80 min of exercise at 70% VO_{2max}. The energy value of EPOC may be increased by higher intensity exercise. Brockman et al. (7) concluded that high intensity exercise, either intermittent or continuous, led to a greater recovery oxygen consumption compared to low intensity exercise. This observation is supported by Gore and Withers (11), who showed that exercise intensity was the major determinant of EPOC.

Several studies have investigated EPOC following moderate exercise (1;3;4;9-11;15;17). However, relatively few investigations (7;13;21) have focused on the comparison of EPOC following high intensity and moderate intensity exercise. In order to make a direct comparison of the recovery oxygen consumption following continuous and interval exercise, it is important to ensure that exercise variables such as total work and duration are as similar as possible and to avoid confounding factors such as post-exercise feeding. Previous designs have utilized supramaximal intensities (13), non-equated work and/or exercise duration (7), post-exercise feeding (13), or different methods of determining EPOC (7;13;21) which make comparison of EPOC results following continuous and interval exercise difficult. One previous investigation (21) did equate work without post-exercise feeding but was published only as an abstract and investigated a relatively short recovery period (~48 min). It was felt this question would benefit from a more thorough investigation utilizing a significantly extended recovery period.

The purpose of the present study was to determine if oxygen consumption was different during the immediate two-hour post-exercise period following continuous and interval exercise protocols where total work was matched and there was no post-exercise feeding. Exercise duration and intensity were selected so as to be appropriate for recreational athletes and fitness program participants.

3.3 Methods

3.3.1 Subjects

Twelve physically active males who were not engaged in specific training volunteered to participate in this study that had been previously approved by the appropriate institutional research ethics board. Each subject provided written informed consent and complied fully with the research protocol. Subject characteristics (mean ± SD) at entry were: age, 30 ± 7 years; height, 179.2 ± 7.5 cm; weight, 86.2 ± 13.1 kg; relative cycling VO_{2max}, 49.7 ± 8.9 $ml \cdot kg^{-1} \cdot min^{-1}$; absolute VO_{2max}, 4.22 ± 0.4 $l \cdot min^{-1}$; and body mass index, 26.8 ± 4.2

3.3.2 Metabolic Measurements

All gas exchange measurements were conducted using a calibrated SensorMedics 2900Z MMC (Yorba Linda, CA) with results averaged every 20 s.

3.3.3 Experimental Protocol

Each subject reported to the laboratory on four separate days (Day 1–4) that were separated by at least 72 h. Day 1 consisted of a graded exercise test (GXT) for the assessment of ventilatory threshold (Tvent) and maximal oxygen consumption (VO_{2max}). Tvent was determined through visual inspection of the exercise data as the power output that caused a systematic increase in the ventilatory equivalent for oxygen ratio (V_E/VO_2) while the ventilatory equivalent for CO_2 was not rising. This point has been described as 'Stage II' by Wasserman (19). Subjects were familiarized with the electro-magnetically braked cycle ergometer (SensorMedics 800S, Yorba Linda, CA) and use of the breathing valve (Hans Rudolph Inc., Kansas City, MO) with noseclip attached. Heart rate (HR) was monitored using a Polar telemetry system (Polar Electro Inc, Woodbury, NY). A 5 min warm-up at 50 watts (W) was followed by workload increases of 25W per min until the subject was unable to continue. Standard criteria for heart rate, respiratory exchange ratio, and plateauing of VO_2 were used to confirm VO_{2max} (18). Minute power output and VO_2 values were used to generate a least-squared regression equation to determine power outputs (PO) corresponding to selected percentages of VO_{2max} that were then used for the experimental exercise challenges.

On Day 2, each subject practiced the interval exercise protocol (INT). The INT protocol was designed to fatigue the subject after seven intervals so that an eighth interval could not have been completed. During the practice sessions, two subjects failed to complete the protocol as planned so the high intensity workload was reduced by 25 W. Two subjects completed the practice with apparent ease, and subsequently their high intensity workload was increased by 15 W. These four subjects then repeated the practice session (following 2 days rest) successfully at the new intensities.

Days 3 and 4 consisted of the continuous exercise protocol (CON) or the INT exercise protocol, which were randomly ordered. Subjects were asked to abstain from strenuous exercise for 36 h before each experimental day. Each participant maintained a record of his dietary intake for the 24 h prior to the first experimental day and then replicated the same intake prior to the second experimental day. The laboratory was kept as quiet as possible during the experiments. Ambient temperature was maintained at 23.5 ± 0.7 °C and each subject wore T-shirt, shorts, and training shoes for both experimental days. The CON and INT protocols each consisted of three phases: pre-exercise; exercise; and post-exercise. The pre and post exercise phases were identical for both INT and CON.

Pre-Exercise Phase

The resting metabolic rate (RMR) was determined during the pre-exercise phase. Each subject was picked up at his home after at least a 12 h fast and transported to the laboratory by WJM at approximately 7:00 A.M. Upon arrival, the subject voided and then body weight was measured. He was then fitted with a HR monitor and head-mount breathing mask (Hans Rudolph Inc., Kansas City, MO) and rested quietly on a bed in the supine position. After at least 15 min of rest, the subject was connected to the MMC for 20 min of baseline data collection. Average heart rate and gas exchange data during the final 10 min were used as baseline. Immediately after baseline values were obtained, the subject was switched to a Hans Rudolph one-way breathing valve and mouthpiece and prepared to perform the exercise phase of either the CON or INT challenge on the electro-magnetically braked cycle ergometer.

Exercise Phase

The exercise phase consisted of a standardized warm-up followed by the INT or CON exercise protocol. The warm-up lasted for 6 minutes and was the same for each subject for both exercise protocols. The warm-up began at an intensity of 50 W, which was subsequently increased each minute until a PO 5 W below 65% of the subjects VO_{2max}. The INT protocol was a 35 min session composed of seven intervals, each consisting of two min at the PO eliciting 90% of VO_{2max} followed by three min at the PO eliciting 30% of VO_{2max}. The CON protocol consisted of 30-32 minutes of exercise at a PO eliciting 65% VO_{2max}. The duration of the CON session was adjusted to match the work output to INT. The work intensity for the continuous exercise treatment was below the ventilatory threshold (Tvent) for all subjects. Gas exchange data were collected continuously throughout exercise.

Post-Exercise Phase

Upon completion of exercise, the post-exercise phase was initiated with the subject being seated in a chair immediately adjacent to the cycle ergometer while gas exchange and HR data were collected continuously. After 10 min, the mouthpiece and breathing valve were removed and the subject drank 125 ml of water. He then quickly re-donned the face-mask used for the RMR measurement and moved to the adjacent bed for the remainder of the 2 h recovery period. Expired gases and HR were continuously monitored for the duration of the recovery period with the exception of two 10 min periods (minutes 60-70 and 90-100 post-exercise) when the mask was removed for subject relief and calibration of MMC.

3.3.4 Analysis of Post-Exercise Oxygen Consumption

Three methods were used to analyze post-exercise oxygen consumption: two EPOC methods and an analysis of variance of total post-exercise oxygen consumption. The one-standard deviation method (1 SD), identified EPOC termination as the time when minute VO_2 values fell to within 1 SD of baseline VO_2 for two consecutive minutes (8;10). The five-minute EPOC method (5 min), identified EPOC termination as the time when the 5 min average of recovery VO_2 equaled the baseline VO_2 (15;16). For both the 1 SD and 5 min methods, the time from the end of exercise to EPOC termination was considered the EPOC duration, and EPOC magnitude was calculated as the integrated area between the recovery minute VO_2 curve and the baseline VO_2 (17).

3.3.5 Statistical Analysis

Comparisons between CON and INT were made using the student t-test for paired groups. Post-exercise total oxygen consumption, respiratory exchange ratio (RER) and HR data were analyzed (in 10 min segments) using a two-way analysis of variance (ANOVA) with repeated measures. When ANOVA revealed significant differences, a post hoc test was used to correct for multiple comparisons (Newman-Keuls test). An alpha level of 0.05 was used to determine significance.

3.4 Results

Baseline values preceding INT and CON treatments were similar for VO_2 (CON: 273 ± 51 $ml \cdot min^{-1}$, INT: 284 ± 54 $ml \cdot min^{-1}$), RER (CON: 0.87 ± 0.07, INT: 0.86 ± 0.06), and HR (CON: 55 ± 9 bpm, INT: 56 ± 9 bpm) indicating that the subjects began the exercise treatments in comparable physiological states. These values were similar to those reported by others (9). Exercise data are displayed in Table 3-1. Despite the attempts to match work for the CON and INT exercise treatments there was a small but statistically significant difference. Total work was higher by 3.63 kJ (<1.0 kcal) in the CON treatment. In addition, we observed greater total exercise oxygen consumption and a higher RER during the INT treatment. The duration of the INT treatment was greater by an average of 4 min.

The student t-test for paired samples revealed that there were no significant differences between the magnitude or duration of EPOC following interval or continuous exercise using either the 1 SD or 5 min methods (Table 3-2). In support, the ANOVA of post-exercise oxygen consumption revealed no difference in recovery values following continuous and interval exercise.

Figure 3-1 displays the 2 h oxygen consumption values (in 10 min segments) for the recovery period following CON and INT. Both CON and INT treatments produced a similar pattern during this period, dropping steadily from the immediate post-exercise elevated levels to near resting levels at the end of the two hour period. The average oxygen consumed during the first 10 min segment of recovery was 597 ± 127 and 594 ± 99 $ml \cdot min^{-1}$ for CON and INT, respectively. The average oxygen consumed during the last 10 min segment of recovery was 281 ± 56 and 287 ± 68 $ml \cdot min^{-1}$ for CON and INT, respectively. The total 2 h post-exercise oxygen consumption was 34.2 ± 5.8 and 36.1 ± 6.1 liters for CON and INT, respectively, a difference of less than two liters.

The similarities between post-exercise oxygen consumption following the two exercise treatments were contrasted by significant differences in post-exercise HR and RER (See Figure 3-2 for RER data). Throughout the 2 h recovery period, we observed significantly higher HR values ($p< 0.05$) and lower RER values ($p< 0.05$) following the INT exercise treatment.

3.5 Discussion

Three methods were used to analyze post-exercise oxygen consumption following the interval and continuous exercise treatments. Each of these methods has been reported previously in the scientific literature, however the duration and magnitude of EPOC was dependent upon the method selected. Regardless of the method used, there were no differences between exercise treatments for EPOC magnitude or duration (Table 3-2). Similarly, the ANOVA revealed no significant difference in total post-exercise oxygen consumption. These results provide compelling evidence that the recovery oxygen consumption is the same following interval or continuous exercise when the total work and duration are similar. These results contradict those published in abstract form from an investigation utilizing apparently similar interval and continuous exercise protocols (21). This abstracted study reported greater EPOC magnitude and duration following interval compared to continuous exercise. However, the duration of interval and continuous exercise, the precise method of EPOC termination, and the point of internal exercise termination were not mentioned in the abstract. All these factors could influence the results and without further information, direct comparison is difficult.

An interesting finding from the data is the effect of EPOC duration on EPOC magnitude. Although the 5 min method produced substantially longer EPOC duration (approximately 80% longer) there was only a small effect (approximately 13%) on EPOC magnitude. This may be understood by referring to the pattern of post-exercise oxygen consumption as illustrated in 3-1. It is clear that the vast majority of increase in post-exercise oxygen consumption occurs during the early phase of the recovery period. As recovery continues, oxygen consumption gradually diminishes, resulting in a reduced increase in magnitude for a standardized increase in duration. Therefore, although the duration of EPOC from the 5 min method was much longer, the resulting increase in EPOC magnitude was relatively small.

A number of past investigations have used continuous exercise protocols similar in intensity and duration to the CON treatment in the present study. Many of these have reported EPOC magnitude results similar to those obtained in this investigation (8;10;11;13). Although EPOC magnitude has been similar, EPOC duration has varied. Using the 1 SD method to determine termination of EPOC after eight healthy young women cycled at approximately 65% VO_{2max} for 30 minutes, Dawson et al. (10) reported EPOC duration of only 13.9 minutes. Chad and Wenger (8), also used the 1 SD method and reported EPOC duration of 128 minutes for a similar protocol. Sedlock et al. (16) had ten male triathletes cycle at 75% VO_{2max} for 300 kcal and observed an EPOC duration of approximately 33 minutes using the 5 min method. As demonstrated in 3-1, as EPOC duration is extended, there is a continual decrease in the contribution to EPOC magnitude.

Whether determined by the 1 SD or 5 min methods, the duration of EPOC following interval exercise in this investigation was less than two hours, an observation in keeping with the findings of Ziegenfuss and Sedlock (21) but not others (2;13). Reports of longer EPOC duration following high intensity exercise have typically resulted from experiments that have included both supra-maximal intensities and post-exercise feeding (2;13). The duration and magnitude of EPOC has been shown to increase exponentially when exercise intensity approaches or exceeds 100% VO_{2max} (12) and perhaps intensities at or over 100% VO_{2max} lead to an extended EPOC. Ingestion of carbohydrate after exercise has been shown to increase the thermal effect of food and produce a higher rate of oxygen consumption (5). It may be possible that the thermal effect of food was increased to a greater degree following high-intensity exercise and could have contributed to the extended EPOC duration.

The magnitude of EPOC that was observed in this study was not as large as has been previously reported in other investigations (3;16). When considered as a percentage of total oxygen consumption during exercise, the magnitude of EPOC was slightly greater using the 5 min method (CON: 8.3, INT 8.5%) than the 1 SD method (CON: 7.3, INT 7.5%). With respect to the influence of EPOC on energy expenditure, there seems to be little advantage in choosing interval exercise over a similar amount of continuous exercise. This point is made after considering that our subjects completed the CON protocol relatively easily, while completion of the INT protocol was difficult and pushed the subjects close to the point of exhaustion.

However, there may be other benefits of high intensity exercise that were not investigated in this study. Our data suggest a differential physiological response to INT and CON exercise. For example, RER values (Figure 3-2) were lower following interval exercise for the majority of the two-hour recovery period. The dip in RER values during the first hour of recovery following both interval and continuous exercise is indicative of CO_2 retention to replenish bicarbonate stores with the more pronounced dip following interval likely due to greater lactate buffering (13). However, the continued significantly lower RER values following interval for the second hour of recovery suggests a greater reliance on fat oxidation and agrees with previous findings (13, 20).

In addition, heart rate was significantly higher throughout recovery from interval compared to continuous exercise, an observation that agrees with a previous report (6). The reason for the increased heart rate is unclear (17), but may be related to higher levels of circulating catecholamines (20). While heart rate and RER are fairly crude indicators of the physiological milieu during recovery, it is tempting to speculate that fat metabolism may be increased during the recovery period following interval exercise. This possibility warrants further investigation.

3.6 Summary

This investigation compared oxygen consumption following continuous and interval exercise protocols that were very similar in terms of duration and total work. Under these conditions, post-exercise oxygen consumption was the same regardless of the exercise protocol. Based upon these results, it is unlikely that high intensity interval exercise offers a greater EPOC benefit than a similar amount of moderate intensity exercise. However, our findings demonstrate other responses (RER and HR) that may indicate differences in post-exercise metabolism following interval and continuous exercise. Further investigation should be performed to evaluate any potential effect on post-exercise energy metabolism.

3.7 Acknowledgement

The authors gratefully acknowledge financial support for this experiment from the Sport Sciences Association of Alberta.

TABLE 3-1. Physiological measurements and duration for continuous (CON) and interval (INT) exercise conditions (n=12).

Treatment	Work (kJ)	VO_2 (liters)	$\%VO_{2max}$	Duration (min)	Avg. VO_2 ($liters \cdot min^{-1}$)	HR (bpm)	RER
CON	291.06* ± 43.01	84.11* ± 10.00	64.5* ± 3.0	31.0* ± 1.0	2.73* ± .31	154 ± 11	0.94* ± .02
INT	287.43 ± 41.95	89.38 ± 9.23	60.5 ± 2.9%	35.0 ± 0.0	2.55 ± .26	156 ± 10	1.04 ± .02

Values expressed as mean ± SD. *Significant difference between conditions, $p<0.05$. Work = total work of exercise, VO_2 = total exercise oxygen consumption, $\%VO_{2max}$ = average exercise oxygen uptake as a percent of maximal oxygen uptake, Avg. VO_2 = average oxygen uptake per minute (Avg. $VO_{2max} = 4.22 \pm .42$ $liters \cdot min^{-1}$), HR = average heart rate during exercise, RER = average respiratory exchange ratio during exercise.

TABLE 3-2. Comparison of two different EPOC methods following continuous (CON) and interval (INT) exercise conditions (n=12).

Method	EPOC Measurement Variable	Condition CON	 INT
1 SD	Magnitude (liters O_2)	6.2 ± 2.0	6.7 ± 1.1
	Duration (min)	50.8 ± 32.4	53.0 ± 19.0
5 Min	Magnitude (liters O_2)	7.0 ± 1.8	7.6 ± 1.1
	Duration (min)	94.9 ± 26.3	88.8 ± 29.1

Values expressed as mean ± SD. There was no difference between CON and INT for either method ($p < 0.05$). EPOC = excess post-exercise oxygen consumption.

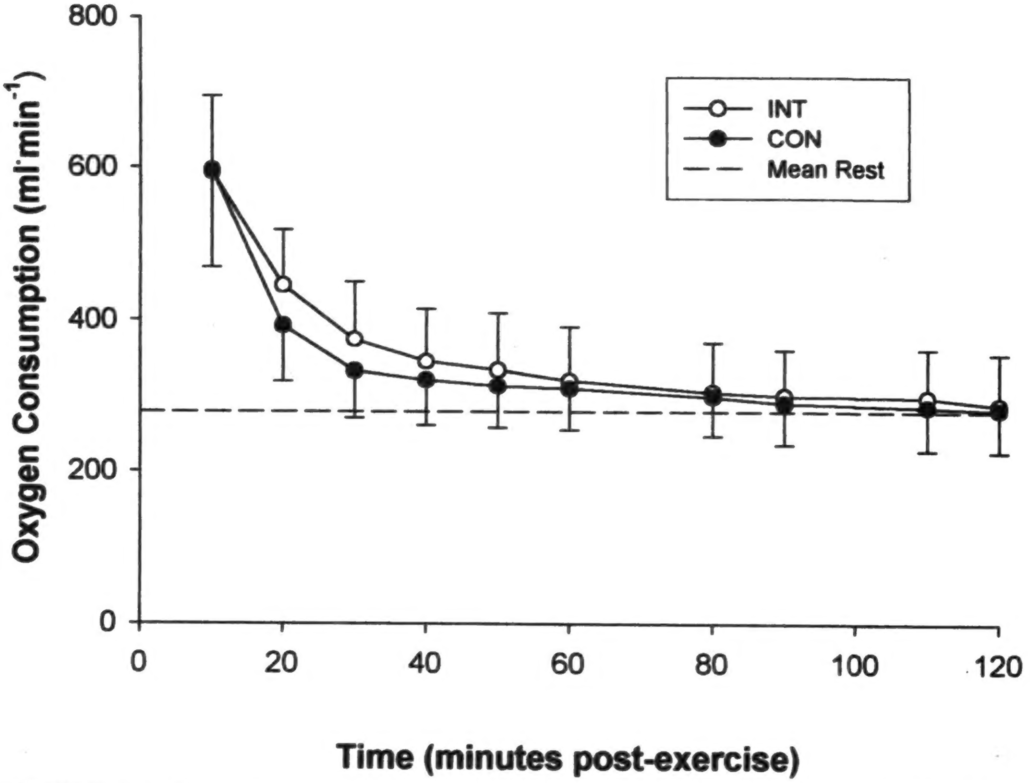

FIGURE 3-1. Oxygen consumption values averaged over 10 min intervals for the 2 h recovery period following continuous (CON) and interval (INT) exercise protocols (n=12). Values expressed as mean ± SD. Mean Rest is the average of the CON and INT resting values.

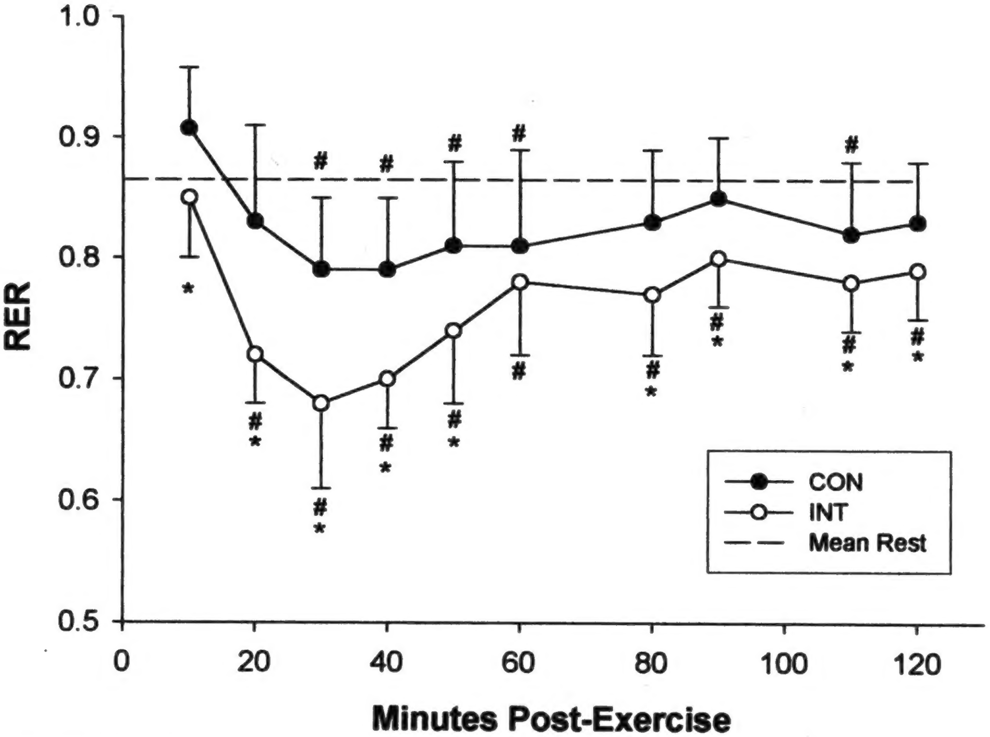

FIGURE 3-2. Respiratory exchange ratio (RER) values averaged over 10 min intervals for the 2 h recovery period following continuous (CON) and interval (INT) exercise protocols (n=12). Values expressed as mean ± SD. Mean Rest is the average of the CON and INT resting values. * Significant differences between groups; # significantly different from Rest within same condition ($p<0.05$).

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CHAPTER 4

5'AMP-ACTIVATED PROTEIN KINASE AND PYRUVATE DEHYDROGENASE ACTIVITY DURING CONTINUOUS AND INTERVAL EXERCISE AND RECOVERY

4.1 Abstract

The activation level of 5'AMP-activated protein kinase (AMPK) and the active form of pyruvate dehydrogenase (PDH_a) were examined in human skeletal muscle to investigate substrate utilization during continuous and interval exercise and recovery. Nine subjects completed two exercise treatments of equal work and duration but different intensities. The interval (INT) exercise treatment consisted of seven 2-min periods at 90% VO_{2max} followed by 3-min at 30% VO_{2max} (with the last 3-min period shortened to 1-min). The continuous (CON) treatment consisted of continuous exercise at 60% VO_{2max}. Metabolic measurements, blood samples and biopsies were taken during rest, exercise and a 1-h recovery period. INT exercise resulted in increased activation of AMPK (INT: 1608 ± 241; CON: 999 ± 92 pmol/min/mg protein) and PDH (INT: 2.73 ± 0.56; CON: 1.49 ± 0.13 mmol/min/kg wet wt.), increased plasma glucose (INT: 117 ± 4; CON: 92 ± 3 mg/dl) and blood lactate (INT: 12.2 ± 0.8; CON: 3.8 ± 0.3 mM), and decreased muscle glycogen (INT: ~53%; CON: ~24% decrease from rest) compared to CON. Reduced RER values during the final 20-min of recovery for INT (0.71 ± 0.02) indicate increased fat utilization compared to CON (0.78 ± 0.01). Recovery PDH_a values (INT: 0.53 ± 0.12; CON: 0.54 ± 0.11 mmol/min/kg wet wt.) were similar to rest (INT: 0.54 ± 0.11; CON: 0.50 ± 0.09 mmol/min/kg wet wt.). Recovery AMPK activity following INT (1284 ± 115 pmol/min/mg protein) remained above CON (876 ± 104 pmol/min/mg protein)

accompanied by increased blood glucose levels and continued reduced glycogen levels. These results support greater carbohydrate utilization during higher intensity interval exercise compared to moderate intensity continuous exercise. In addition, they support increased fat utilization following interval exercise compared to continuous, accompanied by a reduced importance in glucose oxidation following both continuous and interval exercise.

4.2 Introduction

Past research (23;24) has demonstrated that high intensity interval or intermittent exercise has resulted in significantly different respiratory exchange ratio (RER) values during exercise and recovery compared to moderate intensity continuous exercise of equal work. RER values were increased during high intensity exercise and decreased during recovery when compared with moderate intensity exercise. RER is an indicator of substrate utilization, and these results indicate a greater reliance on carbohydrate utilization during high intensity exercise and an increased reliance on fat oxidation during recovery.

Pyruvate dehydrogenase (PDH) is a key enzyme in controlling the flux of pyruvate-derived acetyl-CoA into the Krebs cycle (35). During exercise, PDH activity is positively related to exercise intensity (16), yet research during recovery has only been performed on fish (37) or humans participating in post-exercise carbohydrate-rich feeding (20). The 5'-AMP-activated protein kinase (AMPK) also plays a role in fuel utilization in skeletal muscle cells. AMPK activity has been related to increased glucose uptake (25) and is considered an important component in fatty acid oxidation within the muscle cell (18;46). In human skeletal muscle, AMPK activation has been related to exercise intensity (8;14;47) and substrate availability, including glycogen (14) and blood glucose (27) concentrations. Only two investigations (7;50) have examined AMPK activation during exercise above 80% VO_{2max}, and neither investigated AMPK during recovery. In addition, plasma concentration of non-esterfied fatty acids (NEFA) has been reported to affect utilization (11) and research has demonstrated that higher intensity exercise results in different plasma NEFA concentrations during exercise and recovery compared to moderate intensity exercise (38).

Collectively these results suggest that the activation level of AMPK and PDH, and the availability of specific substrates play a role in determining cellular metabolism. The purpose of this study was to investigate the effect of moderate intensity continuous and high intensity interval exercise of equal duration and work on enzymatic activity and substrate availability during exercise and recovery.

4.3 Methods

4.3.1 Subjects

Nine active but untrained male subjects provided written informed consent to participate in the study. Subject characteristics (mean ± SE) at entry were: age, 25.0 ± 2.0 years; height, 177.7 ± 2.0 cm; weight, 79.8 ± 4.6 kg; relative cycling VO_{2max}, 45.6 ± 2.3 ml/kg/min; absolute VO_{2max}, 3.59 ± 0.2 l/min, and body mass index, 25.2 ± 1.2. Experimental procedures were approved by the Health Research Ethics Board (Panel A) of the University of Alberta.

4.3.2 Experimental Procedure

Subjects reported to the laboratory four times over approximately a four-week period. At least 10 days before the experimental trials, subjects performed an incremental test on an electro-magnetically braked ergometer (SensorMedics 800S, Yorba Linda, CA) to determine VO_{2max} (Parvo Medics TrueMax 2400 MC, Salt Lake City, UT). The linear relationship between oxygen uptake and power output was used to determine power outputs for the experimental trials. At least seven days before the experimental trials, all subjects successfully performed a familiarization session of the interval exercise treatment.

During the following 2-3 week period, each subject reported to the laboratory twice (separated by at least 7-days) to perform the randomly assigned interval and continuous exercise protocols on the electro-magnetically braked ergometer. Subjects recorded activity and diet for the 48-hour period prior to the experimental treatments and abstained from strenuous physical activity and alcohol for the same period. Diet and activity patterns were replicated prior to the second exercise protocol. The experimental protocol is summarized in Figure 4-1. Resting measurements were taken with the subject in a supine position and followed by the exercise treatments. The interval (INT) exercise treatment consisted of seven 2-min periods at 90% VO_{2max} followed by 3-min periods at 30% VO_{2max} (with the last 3-min period shortened to 1-min) for a total exercise duration of 33-min. The continuous (CON) treatment consisted of exercise at a constant moderate intensity (60.0 ± 1.4% VO_{2max}). The two exercise treatments were equal in duration and work and preceded by an identical 5-min warm-up. Immediately following exercise, subjects were seated in a chair for 10-min then returned to a supine position for the remainder of the 1-h recovery period.

The experimental day began with the subject restricting physical activity while reporting to the laboratory at approximately 6:30 A.M (after a 12-h fast). Resting metabolic measurements, including VO_2, respiratory exchange ratio (RER) and heart rate (Polar telemetry system, Kempelle, Finland) were obtained as previously described (24) followed by the insertion of a catheter in the anticubital or basilic vein and the collection of a resting arterialized venous blood sample using the heated-hand technique. Resting muscle was sampled from one of three prepared sites on the vastus lateralis under local anesthesia using the percutaneous needle technique with suction (3). Biopsies were taken from one limb during the first trial and from the contralateral limb during the second trial.

The rating of perceived exertion (RPE) was evaluated during exercise according to the method previously described (4). Metabolic measurements were obtained during exercise, and the first 10-min and final 20-min of the 1-h recovery period. Blood samples were drawn during INT at the end of the 2-min high intensity and 3-min recovery periods of the first (min 7 and 10) and fourth (min 22 and 25) intervals and the end of the 2-min high intensity period of the seventh interval (min 37) and at corresponding times during CON. During recovery, blood was drawn every ten minutes (min 48, 58, 68, 78, 88, 98). Blood samples were drawn into 10cc syringes, with a 200 μl aliquot immediately pipetted into a preservative tube for lactate analysis with the remainder decanted quickly into heparinzed syringes and centrifuged at 2500 rpm for 10-min. The resulting plasma supernatant was frozen for later analysis.

Biopsies were taken during exercise at min 32 (following the sixth 2-min high intensity period during INT) and 26-min into recovery. This recovery time point had previously been determined to be when RER values were at the lowest level following identical exercise protocols (24). Muscle samples were immediately frozen in liquid nitrogen. Exercise biopsy samples were frozen 35.6 ± 1.7 s after the cessation of exercise.

A standard 90-s period was allowed for completion of the biopsy and taping of the area before resuming exercise.

4.3.3 Analytical Techniques

4.3.3.1 Blood Analysis

Blood lactate was determined in duplicate using an automated lactate oxidase method with re-analysis for differences greater than 0.3 mM and re-calibration every 5 samples (Yellow Springs Instrument Model 2300 Analyzer, Yellow Springs, OH). Pre-frozen plasma supernatant was analyzed in duplicate by enzymatic colorimetric procedure for non-esterfied fatty acids (Wako NEFA-C kit, Wako Chemicals USA, Inc., Richmond, VA) and glycerol (Sigma Diagnostics Triglyceride [GPO-Trinder] kit, Sigma Diagnostics, Inc., St Louis, MO) with re-analysis performed with coefficient of variation greater than 20%. Blood glucose was analyzed in duplicate from the remaining supernatant using a Beckman Glucose Analyzer 2 with re-analysis for differences greater than 5 mg/dl and re-calibration every 10 samples (Beckman Instruments Inc., Fullerton, CA). A detailed methodology of the blood collection procedure is available in Appendix B.

4.3.3.2 Muscle Analysis

Two samples, a 40-60 mg sample for AMPK analysis and a 10-30 mg sample for PDH_a analysis were chipped from each biopsy and dissected free of blood and connective tissue under liquid nitrogen. The remaining muscle sample was freeze-dried and then crushed to a powder with any visible connective tissue removed. Muscle glycogen was then measured by a hexokinase method after acid hydrolysis as described previously (17).

PDH_a was measured in muscle homogenates using methods previously described (10;34) with the following modifications to the assay buffer. The concentration of the following reagents was increased: EDTA to 0.72 mM, MgCl_2 to 1.44 mM, and DTT to 1.44 mM. Protein concentration of the homogenates were determined using a commercial kit (Bio-Rad, Richmond, CA).

AMPK activity was assayed from liquid nitrogen immersed biopsies by measuring the rate of incorporation of terminal phosphate of 32P-ATP into the synthetic AMARA peptide (AMARAASAAALARRR; Alberta Peptide Institute, University of Alberta, Canada) at 30 °C (2;21). A detailed methodology of the biopsy collection procedure is available in Appendix B.

4.3.3.3 Statistical Analysis

Data are expressed as means ± SE and the level of significance was set at 0.05. All blood, muscle and perceived exertion results were analyzed using a two-way repeated measures ANOVA. When ANOVA revealed significant differences, a post hoc test was used to correct for multiple comparisons (Newman-Keuls test). All work and metabolic data were analyzed using a 2-tailed paired-samples Student's *t*-test. Due to technical problems, blood lactate and muscle glycogen measurements are not available for one subject.

4.4 Results

4.4.1 Metabolic Data

Resting values for INT and CON were similar ($P>0.05$) for VO_2 (CON: 260 ± 10, INT: 260 ± 10 ml/min), RER (CON: 0.87 ± 0.01, INT: 0.87 ± 0.02), and HR (CON: 65 ± 3, INT: 66 ± 3 bpm) indicating that the subjects began the exercise treatments in comparable physiological states. These values were similar to past investigations (1;6;6;24;41;41).

Exercise values for CON and INT were similar for work (CON: 267 ± 13; INT 266 ± 12 kJ), duration (CON: 33; INT: 33 min), oxygen consumption (CON: 70.7 ± 3.0; INT 73.2 ± 2.5 liters), and oxygen consumption as a percentage of VO_{2max} (CON: 60.0 ± 1.3; INT 62.2 ± 1.4%). Differences did occur for exercise HR (CON: 154 ± 5; INT 158 ± 4 bpm) and RER (CON: .96 ±.01; INT 1.07 ± .01). Perceived exertion values were higher for INT (15 ± 1; 17 ± 1; 19 ± 1) than CON (12 ± 1; 13 ± 1; 13 ± 1) throughout exercise.

Average min-VO_2 for the final 20-min of recovery for both CON (301 ± 15 ml/min) and INT (320 ± 15 ml/min) were similar but significantly greater than corresponding resting values (CON: 260 ±10; INT: 260 ± 10 ml/min). RER values for the final 20-min of the 1-h recovery period were significantly lower following INT (0.71 ± 0.02), than CON (0.78 ± 0.01), and both were significantly lower than corresponding resting values (CON: 0.87 ± 0.01, INT: 0.87 ± 0.02). INT HR values (84 ± 2 bpm) for the same period were significantly higher than CON (74 ± 4 bpm), and both were significantly higher than corresponding resting values (CON: 65 ± 3, INT: 66 ± 3 bpm).

4.4.2 Plasma and Blood Metabolites

During exercise and recovery, the one blood and three plasma metabolites measured produced significant interaction between INT and CON values. For lactate (Figure 4-2), glucose (Figure 4-3) and glycerol (Figure 4-4), INT exercise produced a relatively steady increase in values during exercise followed by a gradual decrease to resting (glycerol and glucose) or near resting (lactate) values by the end of the 1-h recovery period. This is contrasted by CON exercise and recovery results. Blood glucose values remained at resting levels throughout CON exercise and recovery. Lactate values during CON exercise rose slightly during exercise, peaking at min-25 and then quickly returned to resting values by 10-min into recovery. Glycerol values during CON followed a similar pattern to that during the INT but with a blunted response during exercise, and a return to resting levels 30-min into recovery. Plasma NEFA (Figure 4-5) also displayed a different pattern for CON than INT. During exercise, both INT and CON values remained similar to rest. At the completion of CON exercise, NEFA concentrations increased significantly, followed by a slight drop during recovery, although CON values remained above INT for the complete recovery period. NEFA values during recovery from INT exercise did not demonstrate the typical increase at the termination of exercise but remained at resting level throughout the complete recovery period.

4.4.3 Muscle Metabolites and Enzyme Activities

Statistical analysis indicated significant interaction between INT and CON values for PDH_a and AMPK and a significant difference in the main effects for muscle glycogen values. INT exercise resulted in a greater change in all muscle metabolites compared to CON exercise. INT exercise resulted in significant increases in PDH_a (Figure 4-6), a significant reduction in muscle glycogen concentration (Figure 4-7), and a significant increase in AMPK (Figure 4-8) activity. In contrast, CON exercise resulted in no change in glycogen concentration or AMPK activity and a blunted PDH_a response during exercise compared to interval. During recovery from INT exercise, AMPK activity was reduced from exercise but remained above resting and CON values. Glycogen concentration did not increase significantly during recovery from INT or CON exercise and PDH_a activity for both INT and CON returned to resting levels during recovery.

4.5 Discussion

The principal findings of this study are: 1) that high intensity interval exercise increased AMPK activity over rest while moderate intensity continuous exercise of equal work did not; 2) that AMPK activity remained elevated 26-min into recovery from high intensity interval exercise, and; 3) that PDH activity returned to resting level 26-min into recovery following both moderate intensity continuous and high intensity interval exercise.

As exercise intensity increases from moderate to high it has been reported that carbohydrate utilization becomes more predominant (38;42). The results of this investigation, where pre-experimental 48-hour dietary intake and activity were duplicated, support this premise. The enzyme PDH controls the flux of pyruvate-derived acetyl-CoA into the tricarboxylic acid (TCA) cycle and therefore provides information on carbohydrate utilization (35). PDH_a was significantly greater during INT than CON exercise (Figure 4-6) with activation levels during exercise being similar to past investigations (31;34;42). The increased PDH_a, the greater levels of blood lactate (Figure 4-2) and glucose (Figure 4-3), and the greater reduction of muscle glycogen (Figure 4-7) during INT compared to CON, support carbohydrate metabolism being more predominant during high intensity interval exercise than moderate intensity continuous exercise.

During INT exercise AMPK phosphorylation was increased over resting values and CON exercise values (Figure 4-8). The results of this investigation, along with those of recent investigations utilizing high intensity intermittent exercise (50) or exercise protocols of progressive intensity (8) support AMPK phosphorylation being increased with exercise intensity. AMPK activation has been reported to increase GLUT4 translocation to the cell membrane with resulting increased glucose uptake (22). In this manner, increased AMPK activation during interval exercise supports increased carbohydrate utilization during interval exercise compared to continuous.

Many human studies have reported an inverse relationship between muscle glycogen levels and AMPK activation during exercise (8;14;40;48) although the mechanism responsible for the relationship is not known (8). Fujii et al. (14) had subjects cycle at 70% VO_{2max} and reported an increase in AMPKα2 activation following 20 and 60 min of exercise with corresponding decreases in muscle glycogen, whereas 20 min of exercise at 50% VO_{2max} resulted in no significant increase in AMPK α2 activation and no decrease in muscle glycogen. In support, subjects were exercised for 1 h at 70% VO_{2peak} in a glycogen loaded or glycogen depleted state and the authors reported higher AMPK activity in the glycogen depleted state, concluding that AMPK activity is sensitive to the fuel status of the muscle (48). The results of the present investigation would be in agreement with this relationship. AMPK activation was increased during INT when there was a corresponding decrease in muscle glycogen concentration and AMPK activation did not increase during CON when glycogen concentration was not significantly decreased during exercise.

Recent research, however, has identified other factors, including adenine nucleotide concentrations, blood glucose levels, and catecholamines, that may be involved in AMPK activation (48). Measurements of AMPK activation were taken after 5 and 30 min of cycle exercise at ~63% VO_{2peak}. AMPK activation progressively increased from rest to 5 min and again from 5 min to 30 min. As muscle glycogen concentration was not significantly decreased at 5 min, the authors concluded that initial activation of AMPK was due to the AMP/ATP ratio with activation at 30 min being a result of reductions in muscle glycogen (40). However, in studies with patients with McArdles disease, who have a reduced ability to utilize muscle glycogen and therefore have higher than normal levels of resting glycogen, AMPK activation at rest was similar to control subjects despite different glycogen levels. Since blood glucose levels were similar between the groups, it was speculated that glucose availability rather than glycogen concentrations may have influenced activation (27). In addition, when glycogen was added to purified rat liver AMPK, activation level was not altered (32). Therefore, glycogen content would not appear to have a direct effect on AMPK but may play a role in conjunction with other factors (8).

Although the relationship between increased exercise intensity and AMPK phosphorylation appears strong, the relationship between AMPK activation and fat oxidation during high intensity exercise appears to be dissociated. It is well known that AMPK activity has been related to fatty acid utilization (18;46). However, recent reports have demonstrated that fat oxidation is maximized at moderate exercise intensities and diminishes as intensity increases (8;38;42). This occurs even though higher intensity exercise has been demonstrated to increase AMPK activity (8), decrease acetyl-CoA carboxylase (ACCβ) activation (12) and decrease malonyl-CoA concentration (12;29).

Recent evidence has identified factors other than malonyl-CoA that may play a role in the regulation of the transport of activated fatty acids into the mitochondria during exercise in humans (12;28;29;42). Free carnitine, which is required for the function of the enzymes carnitine palmitoyltransferase I (CPT1) and carnitine:acyl-carnitine translocase

(CACT) in transport of acyl-carnitine across the inner mitochondrial membrane, has been reported to decrease with exercise intensity (42). As exercise intensity increases, flux through the enzyme pyruvate dehydrogenase (PDH) would greatly exceed flux through the TCA cycle, resulting in increased acetylcarnitine concentrations and reduced free carnitine availability for fat oxidation (42). Reductions in muscle pH have also been reported to reduce fat oxidation by inhibiting CPT1 (39;42). Blood lactate concentrations during INT exercise were increased compared to CON, and increases in blood lactate have been related to decreases in muscle pH (5). Another possible mechanism is the increased recruitment of less oxidative fast-twitch fibers at higher intensity (8). These conditions may have occurred during INT and may explain why increases in AMPK phosphorylation occurred during exercise utilizing intensities that have been reported to inhibit fat oxidation (38;42).

Plasma NEFA contribution to metabolism has been reported to peak at lower intensities and decrease at moderate and high intensities (38;42). As plasma NEFA utilization is related to concentration (11), the results of this investigation support the reduced importance of these metabolites during exercise, as plasma concentrations (Figure 4-5) remained at resting levels throughout both INT and CON exercise.

Results from past investigations have demonstrated that as exercise intensity increases, recovery RER values decrease (23;24) and fat expenditure increases during the post-exercise period (33). Our investigation supports this with RER values for the final 20-min of the 1-h recovery period for INT exercise being lower than those for CON, and both were lower than corresponding resting values.

In support of reduced reliance on glucose oxidation during recovery, PDH_a activity following both CON and INT returned to resting levels 26 min into recovery. Richards et al. (37) exercised rainbow trout to exhaustion and reported PDH values decreased from near maximal levels at the end of exercise to resting levels within 15 min post-exercise, accompanied by indications of a rapid utilization of lipids (both IMTG and plasma NEFA). Resting level PDH_a during recovery from INT exercise in this investigation was accompanied by increased levels of blood glucose and increased AMPK activation, which, as stated earlier, has been reported to increase glucose uptake (22). These events would indicate favorable conditions for increased glycogen synthesis during recovery while limiting glucose oxidation (47).

Analysis of AMPK activation during recovery provides insight into its means of activation and role in fat oxidation. Three investigations have reported the level of AMPK activation during recovery, with the recovery measurement occurring either 30 min (14), 1-h (49) or 3-h (47) post-exercise. All three studies reported significant decreases in muscle glycogen when recovery measurements were taken, however, only the measurement taken 30 min into recovery (14) demonstrated increased AMPK activation. Only two of these studies reported blood glucose concentrations, with elevated blood glucose occurring with increased AMPK activity at 30 min into recovery (14) but resting levels of blood glucose occurring when AMPK activity was not increased 1-h into recovery (49). Collectively these results suggest that if muscle glycogen and blood glucose are related to AMPK activation, the more important factor may be blood glucose, although it has been speculated that other factors, including decreases in the PCr/Cr ratio may be required to activate AMPK (49). The results of the present investigation demonstrated increased AMPK activation 26-min into recovery from INT exercise accompanied by both decreased muscle glycogen levels and elevated blood glucose concentrations, making any further understanding of these relationships difficult. Multiple measurements of AMPK, blood glucose, muscle glycogen, AMP/ATP and PCr/Cr in the first hour of recovery from high intensity exercise would provide valuable information in understanding the relationship between these factors.

It has been suggested that the activation of AMPK during the recovery period in rodent (36;44) and human studies (14) could mediate the enhanced fat oxidation that occurs following contractile activity and thereby decrease the rate of glucose oxidation (47). The increased activation of AMPK following INT exercise would support the role of this enzyme in enhancing fat oxidation. However, the lack of increased AMPK activation following CON exercise, when RER values also indicated increase fat oxidation over rest, would not. However, it must be remembered that allosteric activation of the enzyme was not measured in this investigation and AMPK may have played a role in fat oxidation during CON exercise and recovery.

The relationship between plasma NEFA concentration and AMPK activation has been previously examined. Increased plasma NEFA concentration for 5 h did not change AMPK activation in rat muscle (30). Our results support this, as during pre-biopsy recovery from continuous exercise, plasma NEFA concentrations were significantly increased over rest, yet recovery AMPK activation remained unaffected. Alternately, AMPK activation remained above resting values during recovery from interval exercise when plasma NEFA levels remained at resting levels.

NEFA oxidation is in large part dependent upon plasma concentration with increased NEFA oxidation occurring with increased concentration (15). As indicated by RER values lower than rest, increased fat oxidation during CON recovery may have been related to increased oxidation of plasma NEFA, as these increased over resting values during early recovery from CON exercise. Significantly lower RER values during recovery from INT compared to CON would indicate even greater fat oxidation during INT recovery. However, increased uptake and oxidation of plasma NEFA was unlikely to be a major contributor as concentration remained at resting levels throughout recovery. This suggests that IMTG may have been a more prominent fuel during recovery from INT than during recovery from CON. Although past research has demonstrated IMTG utilization during recovery (19), recent investigations using moderate intensity exercise (43) and high intensity interval exercise (20) reported no utilization of IMTG during the recovery period. Measurement of IMTG was not performed during this investigation as the distribution of IMTG in untrained subjects, such as ours, is not evenly dispersed in different fiber types (13) and repeated biopsies have displayed large variations in the coefficient of variation (45).

In conclusion, high-intensity interval and moderate-intensity continuous exercise of equal work and duration resulted in different activation levels of PDH and AMPK, and different substrate availability and utilization during exercise and recovery. During exercise the increased activity of these enzymes, glucose availability, and glycogen utilization favored increased carbohydrate utilization during interval exercise. During recovery, resting level PDH activity indicated a reduced reliance on glucose oxidation following both continuous and interval exercise, and increased AMPK activity following interval exercise supported increased fat oxidation compared to continuous exercise recovery.

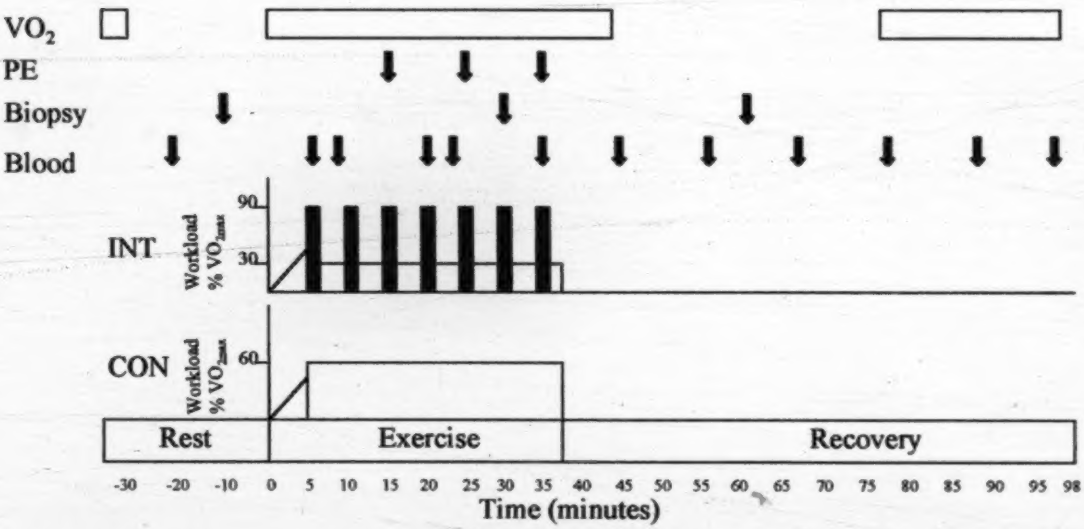
VO_2
PE
Biopsy
Blood
INT
Workload % VO_{2max}
90
30
CON
Workload % VO_{2max}
60
Rest
Exercise
Recovery
-30 -20 -10 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 98
Time (minutes)

FIGURE 4-1. Experimental protocol. INT = interval exercise protocol; CON = continuous exercise protocol; VO_2 = metabolic measurements; PE = perceived exertion; Biopsy = muscle biopsies; Blood = blood sampling.

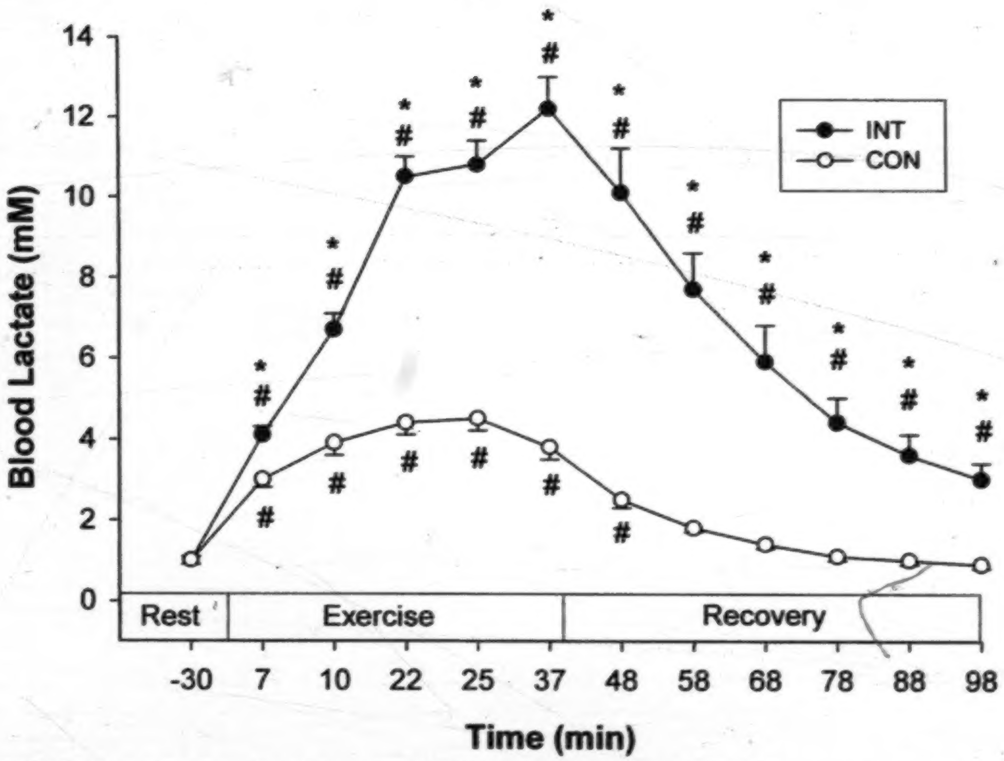

FIGURE 4-2. Blood lactate concentrations for CON and INT during rest, exercise, and 1-h recovery (n=8). Values are means ± SE. * Significant difference between treatments; # significantly different from Rest concentrations within same treatment (P<0.05).

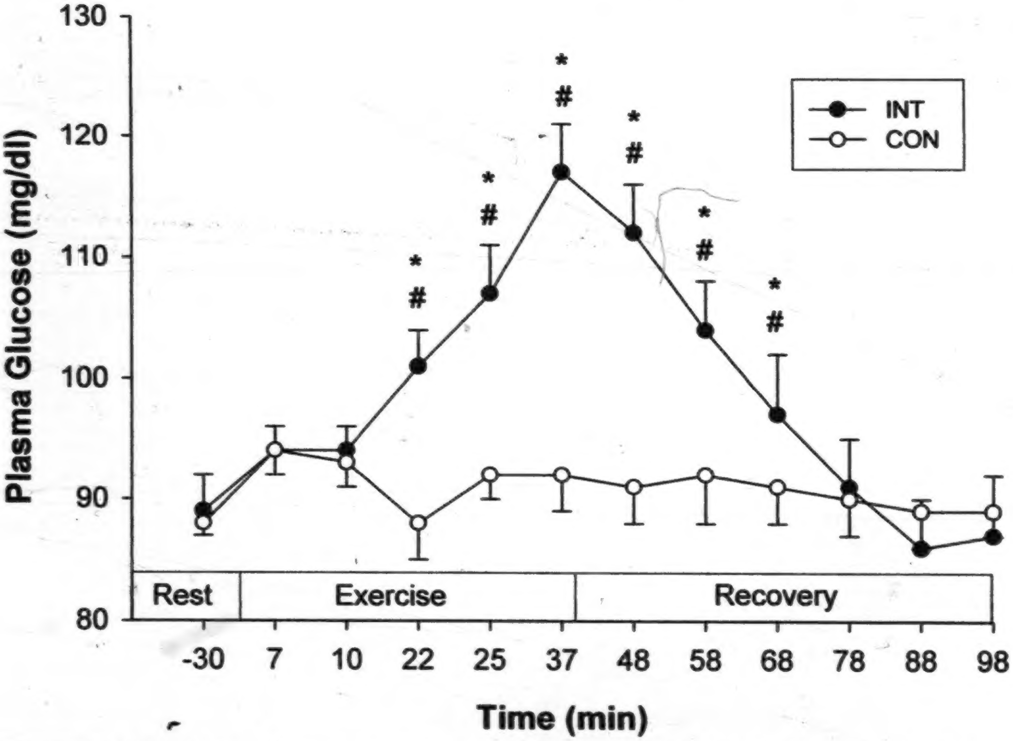

FIGURE 4-3. Plasma glucose concentrations for CON and INT during rest, exercise, and 1-h recovery (n=9). Values are means ± SE. * Significant difference between treatments; # significantly different from Rest concentrations within same treatment ($P<0.05$).

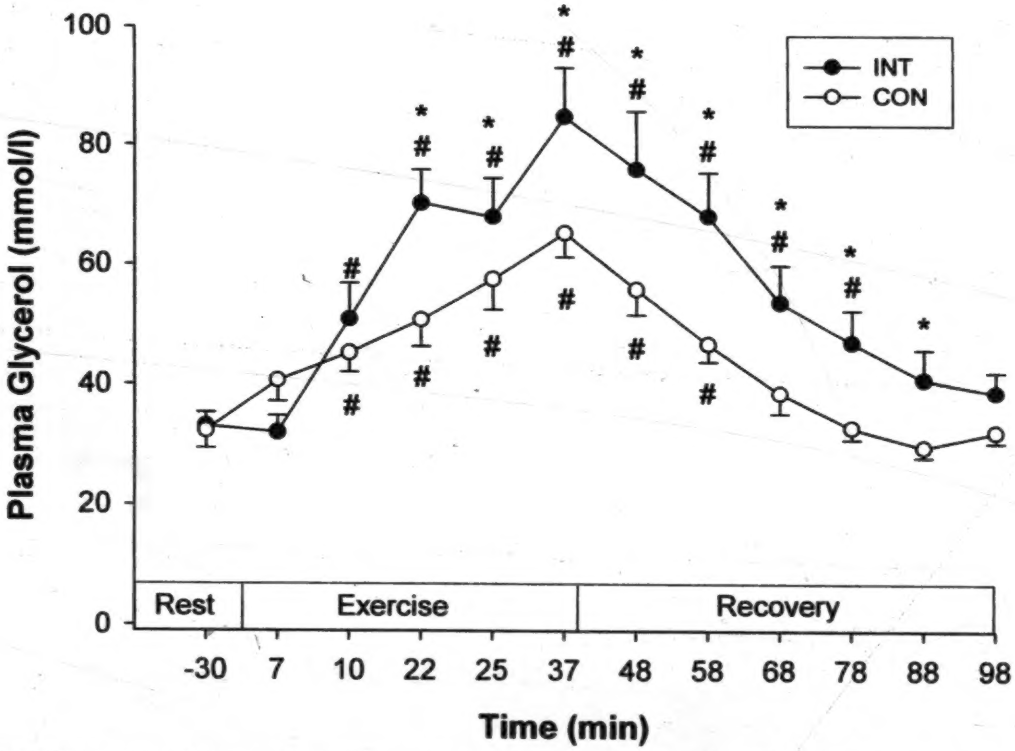

FIGURE 4-4. Plasma glycerol concentrations for CON and INT during rest, exercise, and 1-h recovery (n=9). Values are means ± SE. * Significant difference between treatments; # significantly different from Rest concentrations within same treatment (P<0.05).

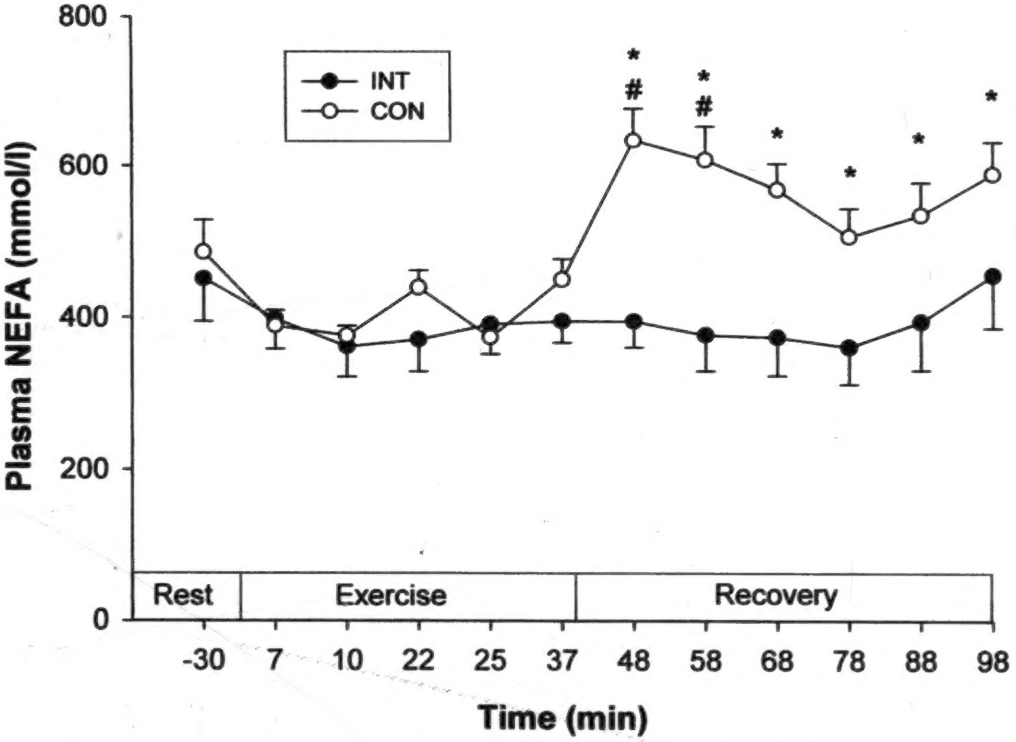

FIGURE 4-5. Plasma NEFA concentrations for CON and INT at rest, exercise, and 1-h recovery (n=9). Values are means ± SE. * Significant difference between treatments; # significantly different from Rest concentrations within same treatments ($P<0.05$).

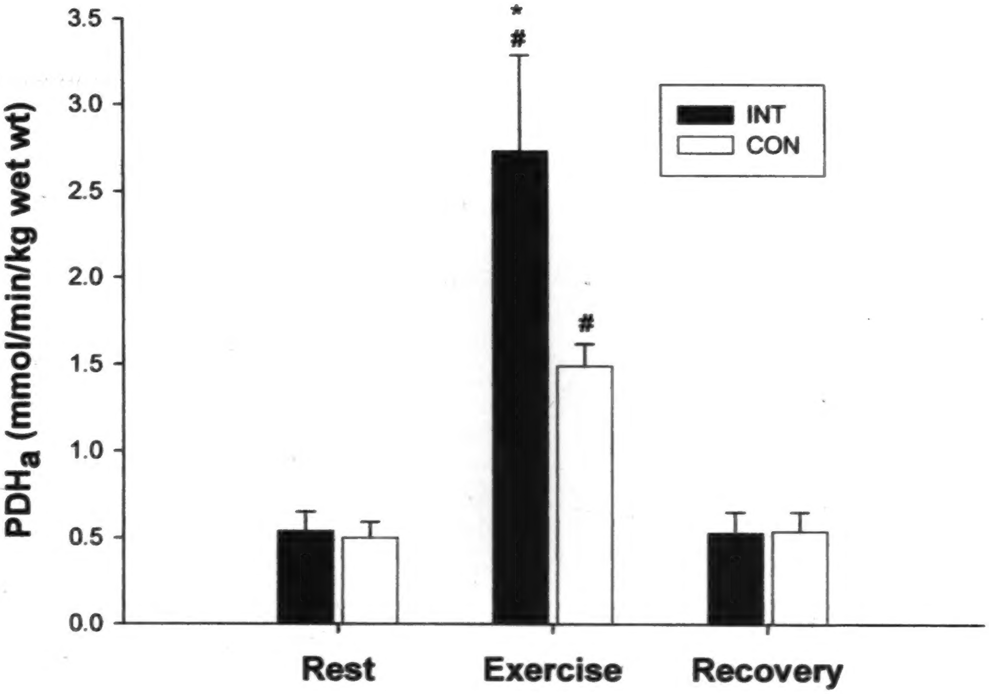

FIGURE 4-6. PDH$_a$ activity for CON and INT during rest, exercise, and 1-h recovery (n=9). Values are means ± SE. *Significant difference between treatments; # significantly different from Rest concentrations within same treatment ($P<0.05$).

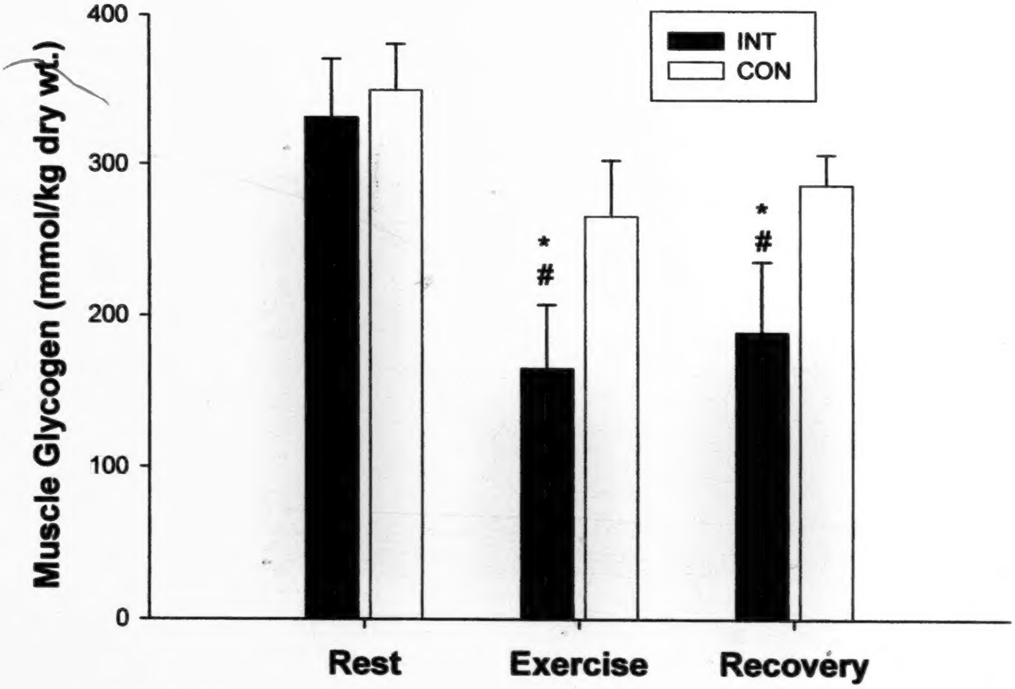

FIGURE 4-7. Muscle glycogen concentrations for CON and INT during rest, exercise, and 1-h recovery (n=8). Values are means ± SE. *Significant difference between treatments; # significantly different from Rest concentrations within same treatment ($P<0.05$).

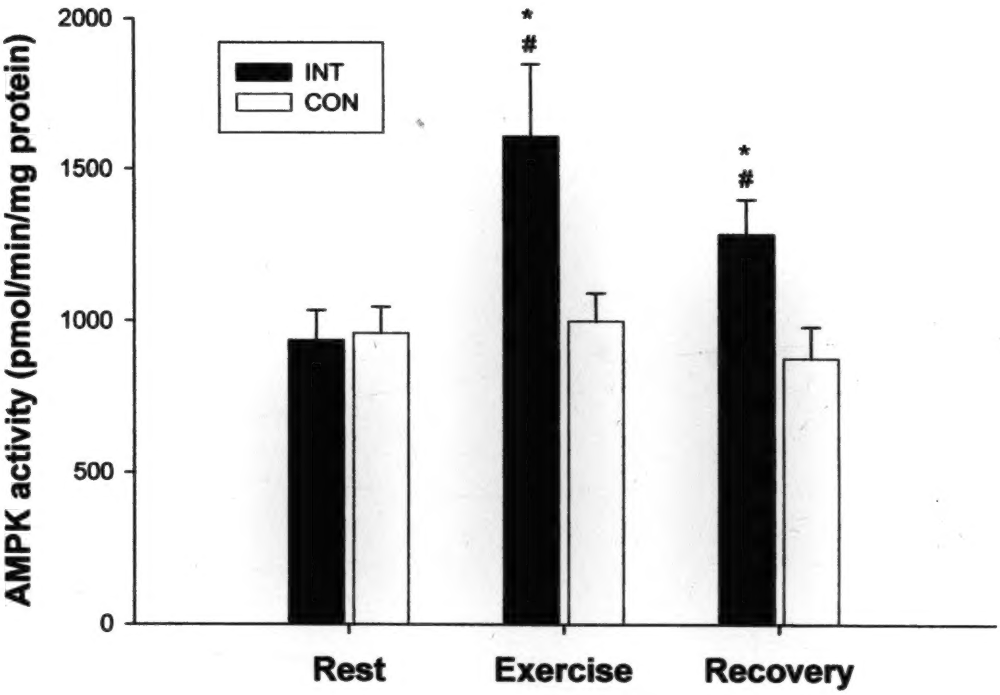

FIGURE 8. AMPK activity for CON and INT during rest, exercise, and 1-h recovery (n=9). Values are means ± SE. *Significant difference between treatments; # significantly different from Rest concentrations within same treatment ($P<0.05$).

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CHAPTER 5

GENERAL DISCUSSION

5.1 Introduction

Cross-sectional studies have demonstrated that men and women who have participated in intense physical activity displayed lower fat percentage, reduced subcutaneous adiposity and lower waist-to-hip ratios compared to those who participated in lower intensity exercise (25;31). Training studies have been more equivocal with some supporting the importance of high intensity exercise for weight loss (4;26) and others showing no effect on body fat (7). The purpose of this thesis was to examine exercise protocols of similar work and duration but different intensities in an attempt to obtain a greater understanding of the various responses elicited during exercise and recovery in terms of energy utilization, substrate utilization and availability, and enzyme activation.

5.2 EPOC Investigation

The first investigation in this thesis included the development of two exercise treatments that were similar in work and duration but dissimilar in exercise intensity and the level of fatigue produced. Interval exercise is a form of training consisting of periods of high intensity separated by periods of lower intensity recovery. This form of exercise was utilized as it provided an effective means of incorporating intensity into training by permitting more minutes at higher intensities than continuous exercise at a high intensity (3). It was decided to use interval exercise (alternating high and low intensities) as opposed to intermittent exercise (alternating supramaximal intensities and complete rest) as it was believed the interval program would be more likely to be used by a recreational athlete or exerciser than the intermittent program due to the stop-start requirement of the intermittent program.

It was hypothesized that if higher intensity exercise had a greater effect on energy balance than moderate intensity exercise of equal work, the negative energy balance should occur or become apparent in the recovery period. In the first investigation, excess post-exercise oxygen consumption was analyzed following continuous (CON) and interval (INT) programs of similar work and duration. Previous research in this area (1;3;11;32) suggested that the higher intensity exercise protocol would lead to greater excess post-exercise oxygen consumption (EPOC). The results of our EPOC investigation contradicted those results and demonstrated that EPOC following continuous and interval exercise treatments of similar work were not significantly different regardless of the methodology used for measuring EPOC. This result was supported by the oxygen consumption values measured in the second investigation (Enzyme/Substrate Investigation) where in the final 20 minute-period of the 1-h recovery, there was no significant difference in total oxygen consumption following the interval or continuous exercise protocols.

Based upon the results of the EPOC Investigation, it appeared unlikely that differences in energy utilization between CON and INT would indicate a greater benefit for interval exercise in regards to energy balance. However, there were physiological differences that existed in the recovery period. Heart rate during recovery from interval exercise was higher than during recovery from continuous exercise. In addition, the respiratory exchange ratio (RER) was lower during recovery from interval than continuous exercise. Both these findings were in agreement with past investigations (11;16). Based upon these differences, it was speculated that the possible benefit of higher intensity exercise may not be a difference in post-exercise oxygen consumption or energy utilization, but rather a shift in fuel utilization from an approximate equal mixture of carbohydrates and fats at rest (21;22) to a greater utilization of fats during recovery from higher intensity exercise compared to moderate intensity continuous exercise.

5.3 Enzyme/Substrate Investigation

The second investigation in this thesis attempted to understand the differences in fuel availability and utilization during exercise and recovery from interval and continuous exercise, and to understand the mechanisms involved in these changes. Exercise protocols similar to those in the first investigation were used with only minor adjustments being made based upon observations and results from the EPOC Investigation. It was determined that the variables to be investigated would include substrate availability and the activity of enzymes of importance to the utilization of these substrates. Substrates measured included those present in the blood (plasma [glycerol], plasma [NEFA], plasma [glucose], and blood [lactate]) and in the muscle (muscle [glycogen]). Although the measurement of muscle [IMTG] would have provided important information in this investigation, past research has demonstrated difficulties with this assay. These include the difficulty in differentiating extra-myocellular triacylglycerols (EMTG) from IMTG in biopsy samples, and fiber-type differences in IMTG concentration in untrained subjects. These aspects have been reported to make the results of this procedure highly variable (27) and were therefore not included in our analysis.

The two enzymes investigated were pyruvate dehydrogenase (PDH) and 5'AMP-activated protein kinase (AMPK). PDH controls the flux of carbohydrate based acetyl-CoA into the tricarboxylic acid (TCA) cycle (18). AMPK has been speculated to be involved in regulation of numerous cellular functions during exercise and recovery in human muscle, including fat oxidation, glucose uptake, and glycogen metabolism (12). Each subject completed a record of physical activity and diet for the two-days prior to his first exercise treatment. This information was used as a blueprint to replicate nutritional intake and activity before the second to ensure the subjects were in comparable physiological and nutritional states prior to both treatments. Similar values in resting metabolism, RER, and blood and muscle substrate concentrations provided evidence that this objective was achieved.

The principal findings of this study were: 1) that high intensity interval exercise increased AMPK activity over rest while moderate intensity continuous exercise of equal work did not; 2) that AMPK activity remained elevated 26-min into recovery from high intensity interval exercise, and; 3) that PDH activity returned to resting level 26-min into recovery following both moderate intensity continuous and high intensity interval exercise. It was determined that interval and continuous exercise of equal work and duration resulted in different activation levels of PDH and AMPK, and different substrate availability and utilization during exercise and recovery. During exercise the increased activity of these enzymes, glucose availability, and glycogen utilization indicated increased carbohydrate utilization during interval exercise compared to continuous. During recovery, resting level PDH activity indicated a reduced reliance on glucose oxidation following both continuous and interval exercise, and increased AMPK activity following interval exercise supported increased fat oxidation compared to continuous exercise recovery. These results provide a greater understanding of the role of different intensities and exercise protocols on enzyme activation, energy balance, weight loss, and other clinical applications.

5.4 AMPK Activation

Research on the enzyme AMPK has increased in recent years as evidence accumulates as to its importance. Numerous methods have been used to activate AMPK, including exercise and various pharmaceutical methods, including 5-amino-4-imidazolecarboxamide riboside (AICAR), metformin, leptin, etc. Research on the acute effects of AMPK activation have demonstrated its effect on phosphorylation of acetyl-CoA carboxylase (ACCβ), fat oxidation, glucose uptake, enhanced insulin sensitivity, and glycogen metabolism (12). In addition, research into the effects of chronic activation of AMPK has provided insight into its possible role in gene transcription increases in proteins related to glucose metabolism (8) and the mitochondria (2;17) as well as the possible effect of chronic AMPK activation on performance enhancement (13) and clinical considerations (19;28).

Chronic activation of AMPK with AICAR in rats has resulted in increases in the cellular content of GLUT4 protein, hexokinase activity (8), increases in uncoupling protein–3 (UCP-3), and increases in the activity of mitochondrial enzymes citrate synthase, and 3-hydroxylacyl-CoA-dehydrogenase, two enzymes that are known to increase in proportion to mitochondrial volume density (2;17;24). Zong et al. (33) examined the role of AMPK in mitochondrial biogenesis by studying transgenic mice expressing a dominant-negative mutant of AMPK (DN-AMPK). Administration of βguanidinopropionic acid (GPA), a creatine analog which leads to reductions in intramuscular ATP/AMP and PCr/Cr ratios (which activate AMPK), resulted in mitochondrial biogenesis in control mice but not in DN-AMPK mice, leading the authors to conclude that AMPK is a critical regulator in initiating mitochondrial biogenesis. These results suggest that AMPK activation is involved in gene transcription and related to some of the biochemical adaptations that take place as a result of exercise training (12;29).

Research examining the effect of training on AMPK activity also supports the importance of AMPK in this area. Frøsig et al. (5) had eight young men perform supervised one-legged knee extensor endurance training for three weeks with biopsies taken before training began and 15 h after the last training session. Various proteins associated with the different isoforms of AMPK were increased along with basal AMPK activity. In a study investigating differences in AMPK content and response to exercise in trained and untrained subjects, it was determined that trained human skeletal muscle had increased AMPKα1 protein levels and that during exercise, trained subjects had a blunted AMPK response (14). This blunted AMPK response resulted in the authors speculating on the role of AMPK activation in bringing about the various changes of endurance training. They suggested that performance and training-induced cellular adaptations are dependent upon a progressive increase in training amount and intensity and that the observation that AMPK activation is blunted in trained subjects compared to sedentary subjects working at the same relative intensity may explain this phenomenon considering the possible role of AMPK in gene expression. These results indicate that AMPK activation is not a marker of total energy flux during exercise but a result of changes in the energy charge induced by exercise (14). Therefore, a requirement for the level of training stress that might bring about the proposed positive effects of training may be determined by the level of AMPK activation the exercise protocol achieves.

A recent review by Ruderman and Prentki (19) has focused on the likely role AMPK may play in patients with metabolic syndrome, where symptoms typically include insulin resistance, hypertension, and obesity. Increasing evidence indicates that a common factor in these symptoms may be a dysregulation of cellular lipid metabolism correlating with excessive stores of intracellular triglyceride (ITG). The excessive accumulation of ITG is generally viewed as a marker of insulin resistance and cellular dysfunction rather than a causal factor. The authors stated that recent research indicates that impaired insulin action is linked to malonyl-CoA accumulation and a redirection of glucose and fat metabolism to lipid esterification processes (19). The authors propose that a common causal factor could be the dysregulation of the AMPK/malonyl-CoA fuel-sensing and signaling network likely due to a decreased level of AMPK activation or a lack of activation all together (19). This review emphasizes the important role AMPK activation may play in various clinical manifestations.

Various pharmaceutical methods of activating AMPK have been discovered and utilized in research. However, the prototypical activator may be exercise, which has been identified to activate AMPK, lower the concentration of malonyl-CoA and alter intracellular lipid metabolism in muscle, liver and adipose tissue (15;19).

Collectively this research indicates that acute and chronic activation of AMPK by exercise and/or pharmaceutical methods appears to have important applications in clinical and performance terms. One question then becomes what intensity of exercise will activate AMPK to the greatest degree and for the longest period of time? Our investigation, utilizing two exercise protocols of different intensities but equal work and duration, provides valuable information in this area. The continuous exercise treatment utilized in our investigation had subjects exercise at a power output selected to elicit approximately 60% VO_{2max}, an exercise treatment not unlike many continuous exercise programs utilized by recreational athletes and people using exercise as a weight loss tool. During continuous exercise and recovery the level of AMPK activation did not change from resting levels and these results would seem to indicate that a higher intensity of exercise would be required to obtain the stated benefits of chronic activation of AMPK.

It must be remembered that the assay used to measure the activity of AMPK only measures the phosphorylation state of the enzyme and not the allosterically regulated activity. For example, when six healthy untrained subjects participated in a 12-week training program at 40% VO_{2max} three times weekly, results showed an increased capacity to oxidize IMTG at rest, and increased fat oxidation and IMTG utilization during exercise (20). The authors speculated a possible mechanism was the chronic downregulation of ACCβ, potentially leading to lower malonyl-CoA concentrations and less inhibition of CPT1, factors, which have been linked to AMPK activation.

Therefore, it can not be dismissed that chronic activation of AMPK may occur at intensities lower than those used in our investigations. However, it would seem reasonable that if acute and chronic activation of AMPK is related to the many positive clinical and performance improvements, that exercise that activates AMPK would be more beneficial at bringing about these changes than exercise that does not. In addition, as stated earlier, it has been suggested that activation of AMPK likely depends on the degree of change in the energy charge, which has been demonstrated to occur to a greater degree with increased exercise intensity (9). The high intensity interval program utilized in our investigation increased activation of AMPK during exercise and 26-min into recovery. This is supported by past research that demonstrates that AMPK activation is positively associated with exercise intensity (6;23;30). The determination that this relationship exists when exercise work and duration are matched, strengthens the validity of this relationship.

5.5 Summary

This thesis was initiated in an attempt to determine if high intensity exercise protocol of equal work and duration as a continuous moderate exercise protocol would result in any measurable change in energy balance during recovery. When no difference was found, the focus then became to look at differences in post-exercise substrate utilization. The results of the second investigation support the premise of greater fat oxidation occurring following INT compared to CON exercise. In addition, this increased fat oxidation may be at least partly due to greater activation of AMPK during exercise and recovery for INT compared to CON. However, the determination of the effect of interval exercise on AMPK activation both during exercise and recovery, and the growing research on the positive effects of acute and chronic activation of AMPK, have increased the scope of discussion. It has been stated that intense interval exercise can be easily endured by the average person and it may be an important tool to bring about changes in body composition related to weight control (10). It may also be considered that high intensity interval exercise should possibly be considered in the treatment of other metabolic syndromes, including insulin resistance and hypertension, that have demonstrated to be affected by chronic activation of AMPK. In addition, our results and others (6;23;30) demonstrate that for continuous moderate intensity exercise to bring about phosphorylation of AMPK, an intensity of greater that 60% VO_{2max} should be used.

5.6 Future Directions

There are numerous future directions for research in this area. The identification of the lower level of intensity and/or glycogen depletion that may be required for AMPK activation could provide valuable information in the prescription of training programs designed to improve fitness and body composition. Also, as the physiological requirement of exercising at 90% VO_{2max} may prove to be too strenuous for many exercisers, the effect of interval programs of various intensities on AMPK activation would provide valuable information in this area. Regarding AMPK activation during recovery, multiple measurements during the first hour post-exercise would help clarify the relationships between various factors and AMPK activation. In addition, training programs utilizing different exercise intensities and duration are required to increase our knowledge on the most effective ways of bring about the positive changes to basal AMPK activation levels and the clinical and performance improvements linked to chronic AMPK activation.

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APPENDIX A

PILOT WORK REQUIRED FOR THESIS

Criteria for the development of Mode, Intensity and Duration of the Continuous (CON) and Interval (INT) exercise treatments

Pilot work was performed for developing mode, intensity and duration of the exercise treatments. The criteria for the exercise treatments were as follows:

1. That the exercise be performed on a common mode of exercise equipment utilized by typical people using exercise as a weight loss tool.
2. That the mode of exercise allow easy and accurate calculation of the amount of work performed and eliminate recalculations required by fluctuations in subject's body weight.
3. That the duration of the exercise treatments be approximately 30-35 min, a common duration used by people exercising for weight loss.
4. That the CON exercise treatment be similar to a typical mono-intensity exercise session utilized by an average person using exercise as a weight loss tool and therefore be at an intensity below ventilatory threshold.
5. That the INT exercise treatment be achievable by an average exerciser but that it also push the exerciser close to exhaustion. It was hoped that the subject would be able to perform the required number of intervals but that they would be unlikely to perform an additional interval.

6. That the INT intensities would be equally above and below the CON intensity, and that the resting phase of the INT session be of a relatively low intensity but not be complete rest.
7. That the high-intensity and low-intensity periods of INT be relatively equal in duration.

Pilot work, involving three volunteers, was undertaken to determine the exercise intensity and duration to be used for the CON and INT exercise treatments. It was decided that 60% VO_{2max} would be utilized as the work intensity for CON. It was felt this intensity was difficult enough that it would be regarded by the subjects as a "moderate" intensity workout, however, the intensity would most likely be below ventilatory threshold of all subjects. This was confirmed by metabolic data from the pilot subjects.

It was found that when pilot subjects performed exercise work below an intensity eliciting 30% VO_{2max}, subjects reported a feeling of "no work" being performed. Therefore a minimum intensity of 30% VO_{2max} for the low-intensity period of INT was established. As it was desired that the high intensity be equally above the CON intensity as the low intensity was below it, it was determined that 90% VO_{2max} would be the upper intensity and 30% VO_{2max} the lower intensity of the INT treatment. A number of arrangements of these two intensities were attempted to try to determine which would allow the subject to complete approximately 30-35 min of exercise. Pilot testing of intensities included:

- 2 min at 90% VO_{2max}, followed by 2 min at 30% VO_{2max}
  - This arrangement provided the subjects with too little time to recovery between high intensity intervals. The maximum number of intervals completed was five, or twenty-five minutes.
- 3 min at 90% VO_{2max}, followed by 3 min at 30% VO_{2max}
  - Three minutes proved to be too long to sustain at the high intensity. The average number of intervals completed was four, or 24 minutes.
- 2 min at 90% VO_{2max}, followed by 3 min at 30% VO_{2max}
  - This format worked well, with all three pilot subjects being able to complete seven intervals (35 minutes) but unable to complete another interval.

Based on this pilot work the mode, intensities and duration of INT and CON were established.

Methodology of Venous Blood Sampling

Pilot work was performed to determine the difference in concentrations of specific substrates between arterial and venous blood during interval exercise. WJM performed an INT session while arterial blood was collected from the radial artery, and venous blood was collected from the antecubital vein of the same arm. Due to the differences found at end-exercise between arterial and venous blood (Table A-1), it was concluded that a method to achieve arterial blood would be required. It was first discussed to obtain blood from the radial artery and femoral vein. In discussion with Dr. Heigenhauser of McMaster University, a scientist who was very familiar with these techniques, he stated that these they are difficult techniques to use even for an experienced team. He also stated that measurement variations of blood glucose and non-esterfied fatty-acids (NEFA) would likely be greater than arterial-venous differences.

It was then suggested using an arterial venous catheter to collect blood from the radial artery. The doctor who would be performing the procedure during the study advised us that the success rate with this procedure was only approximately 70%, considered too low to be of use in this study. It was finally decided that the best method of obtaining arterial blood was to use the "heated hand" technique, a method that resulted in the collection of arterialized venous blood in past investigations.

Biopsy Methodology

This investigation required at least 80-85 mg of muscle tissue from each biopsy taken for the various assays to be performed. The most common methodology utilized in our laboratories produced approximately 30-60 mg of tissue. Therefore, pilot work was performed to determine a more effective method to obtain the required volume of muscle tissue in a quick effective manner. In discussions with Dr. Reid, a doctor who had performed many biopsies using the suction technique, we were directed to practice our technique on a raw piece of meat (roast). The primary investigator and the doctor who performed all the biopsies in the study refined their technique in this manner.

One of the primary questions to be answered by this session was what techniques would maximize the muscle harvest. Numerous attempts demonstrated that when up to three suction-assisted biopsy clips were performed, there was a continual increase in the size of the biopsy with every clip. However, using more than three clips resulted in no noticeable increase in the biopsy size. Therefore, it was decided that three clips would be used to obtain the biopsies in this study. In addition it was determined that the 5-mm biopsy needle yielded the largest biopsies. Smaller needles (4-mm) yielded a biopsy of similar length but of smaller diameter and larger needles (6-mm) yielded biopsies of greater diameter but shorter length.

In addition, during this session different suction set-ups (size of syringe, length of hose, size of tip, etc) were tested. It was determined that the best tip for suction purposes was a blue pipette tip with the last 1-2 cm clipped off. It was also determined that the shorter the tube between the syringe and the tip of the suction set-up, the greater the suction. However, if the tube was too short it would not allow the required distance between the two people performing the biopsy. It was thus determined that the optimal suction tube length was 35-40 cm. It was also determined that a 10cc syringe provided greater suction than syringes of lesser volume. No syringes of greater volume were experimented with. Plastic tie downs (as opposed to metal clamps) proved more effective at creating an airtight seal at the tip and the syringe with two tie downs at both ends being more effective than one. In addition, it was recommended by the doctor, that because of the risk of transferring contamination from subject to subject, that a new suction set-up be used every testing day.

Table A-1: Differences in arterial and venous blood during interval exercise.

	Pre exercise Blood glucose (mmol/dl-1)	Pre-exercise NEFA (mmol/l-1)	End-exercise Blood glucose (mmol/dl-1)	End-exercise NEFA (mmol/l-1)
Arterial Blood	103.5	.480	98.0	.598
Venous Blood	103.5	.406	94.0	.477

APPENDIX B

EXTENDED METHODOLOGY FOR SECOND INVESTIGATION

EXPERIMENTAL DESIGN

Subjects

Nine moderately active men (age, 25.0 ± 2.0, years; height, 177.7 ± 2.0 cm; weight, 79.8 ± 4.6 kg; relative VO_{2max}; 45.6 ± 2.3 ml/kg/min, absolute VO_{2max}; 3.6 ± .2 L) were recruited for the study. Approval was obtained from the Health Research Ethics Board (Panel A) of the University of Alberta. Subjects were provided with written Informed Consent after having had a thorough orientation of the experimental procedures and explanation of the attendant risks associated with the study. Individual data were coded for anonymity and subjects retained the right to withdraw from the study at any time without consequence.

Pre-experimental protocol

Subjects reported to the laboratory on four separate days (Day 1-4) each separated by at least 72-h. Day 1 and Day 2 involved pre-experimental protocols and Day 3 and 4 involved experimental protocols.

Day 1

Day 1 consisted of a graded exercise test (GXT) for the assessment of ventilatory threshold (Tvent) and maximal oxygen consumption (VO_{2max}) using a ParvoMedics Truemax (Salt Lake City, UT) metabolic measurement system. Gas exchange measurement results were generated every 20 s. Volume and gas analyzers were calibrated before all tests. Flow was measured by a Hans Rudolph 3813 heated pneumotach and calibrated using a 3-liter syringe according to manufacturer's instructions. Gas analyzers (Paramagnetic oxygen analyzer, Infrared, single beam, single wavelength carbon dioxide analyzer) were calibrated with room air, and gases of authenticated concentration (PRAXAIR) were used for pre-test calibration and checking for post-test drift. Tvent was determined through visual inspection of the exercise data as the power output that caused a systematic increase in the ventilatory equivalent for oxygen (Ve/V_{O2}) while the ventilatory equivalent for carbon dioxide (Ve/V_{CO2}) was not rising. This point has been described as 'Stage II' by Wasserman.

Subjects were familiarized with the cycle ergometer (SensorMedics 800S, Yorba Linda, CA) and use of the breathing valve (Hans Rudolph Inc., Kansas City, MO) with noseclip attached. Heart rate (HR) was monitored using a Polar telemetry system (Gay Mills, WI). A 5-min warm-up at 50W was followed by an increase of 25W per min until the subject was unable to continue. Standard criteria for heart rate, respiratory quotient and oxygen consumption plateauing were used to ensure achievement of VO_{2max}. Minute power output and VO_2 values were used to generate a least-squared regression equation to determine power outputs (PO) corresponding to 30% and 90% VO_{2max}. These PO were the workloads for the interval (INT) exercise treatment.

Following is a description of the process used to develop the INT and CON workload intensities. Minute power output and VO_2 values were used to generate a least-squared regression equation (Table B-1). Using this data, a simple regression analysis was generated. The resulting regression equation (Table B-1 data example: $Y = -36.046 + 95.684 X$) was then used to determine the P.O. required for the subject to be exercising at

90% and 30% VO_{2max}. For example, if maximal oxygen consumption for the subject was 4.010 liters, than 90% of that would be 3.609 liters and 30% would be 1.203 liters. Adding these values into the regression equation provided the P.O required to achieve these values. For example, the P.O. (W) for 90% equals -36.046 + 95.684 x 3.609 which equals 309 W. The P.O. (W) for 30% equals -36.046 + 95.684 x 1.203 which equaled 81 W. Due to the difficulty of performing the high intensity work and the relative ease of performing the low intensity work, 90% values were rounded down (309 to 305W) and 30% values were rounded up (81 to 85W) to the nearest 5 W.

Determination of the P.O. required for the CON program was achieved by determining the total amount of work to be performed during the INT program (minus the warm up which was identical for INT and CON) and dividing by the duration of CON, which was identical to the duration of the INT treatment (33 minutes). For example, Table B-2 displays an example of the workloads for an INT program. The total work performed was calculated by adding the total work of the high intensity intervals (high intensity intervals work = 4270W) and the low intensity intervals (low intensity intervals = 1615 W) (4270 + 1615 = 5885 W) and subtracting the work loss due to the 1.5 min (min 33 and half of min 34) stoppage of time required for the taking of the exercise biopsy (85W + 42.5W = 127.5 W). This example provides the total work of INT as being 5757.5 W.

The calculations of CON P.O would be as follows. Dividing the total work of INT (5757.5W) by the CON exercise duration (31.5-min as there are 1.5 minutes required for the exercise muscle biopsy) provides the work required per minute during CON (5757.5 divided by 31.5 equals 182.8W). As the cycle ergometer could not be programmed to

182.8W, the CON program was developed using 180W and 185 W. To match work with INT, the subject would perform 17.5 minutes at 185 W (equaling 3237.5 W) and 14 minutes at 180 W (equaling 2520W) (3237.5W + 2520W = 5757.5W). The 14 minutes at 180W would be equally dispersed throughout the 33-minute exercise session.

The 5-min warm-up was developed as follows. The first minute would be at 50 W. The last minute would be at a workload 5W below the lowest watt used for the CON exercise program. The watts for the second, third and forth minutes would be equal divisions of the difference between the first and fifth minute.

Day 2

Day 2 consisted of a practice of the INT exercise challenge (INT-p) to ensure subjects could tolerate the procedure. Subjects reported to the lab at a time during the day that was convenient for them. Subjects wore shorts, T-shirts and runners and were fitted with a heart rate monitor (Polar telemetry system, Gay Mills, WI). The INT-p process was fully explained to the subjects prior to the practice. The subjects were then put through the full 38-min INT program with the following differences from the experimental protocol. During the INT-p the tension was not lowered during the period of time when the exercise biopsy was to be taken during the INT exercise session, as the purpose of this session was to determine if the subject could tolerate the INT program. One other difference was that during the INT-p, after finishing the last interval, subjects were not brought off the bike and sat on a chair, but were allowed to stay on the bike and pedal until full recovery had been achieved. This session provided an accurate assessment of the subject's ability to make it through the INT exercise session. Subjects who could not complete the INT-p session, had the intensities adjusted downward appropriately.

Subjects who completed the INT-p with too much apparent ease had their intensities adjusted upward appropriately.

This visit was also used to further explain the process that would be followed when the subjects reported to the lab for Day 3 and 4. The laboratory was setup for the full experiment and the subject could be walked through the complete process. In addition, at this time the primary investigator had been through the full INT and CON exercise treatments, including metabolic measurements, blood and biopsy samples, and could more accurately describe the procedures involved, and the recovery from the sessions.

Subjects received a package designed to assist in the preparation for Day 3. Prior to Day 3, subjects adhered to the following guidelines. Subjects recorded their nutritional intake and physical activities for the preceding two days, with no nutritional intake (except water) for the prior 12-h period, and no strenuous exercise or alcoholic consumption for the prior 48 hours. These procedures were duplicated prior to Day 4 with the subjects replicating their diet and physical activities from their Day 3 records. Also at this time, subjects were scheduled in for their Day 3 and 4 visits to the lab during which the INT and CON exercise treatments would be administered. Lab times were limited by the availability of the lab, doctor, nurse, support staff and subject.

Laboratory set-up

The main concern when setting up the research area was to create a working space that provided space for five different functional areas (biopsy collection, biopsy management, blood collection, blood management, and metabolic cart management) without the different sections interfering with each other. This was accomplished by positioning the blood collection, the biopsy collection and the metabolic cart on different sides of the gurney on which the subject lay or the bike on which the subject exercised (Figure B-1).

The function of the different areas was as follows:

- Biopsy Collection Section
    - The doctor worked in this area to: prepare the subject for the biopsy surgery; take the biopsies; and manage the incisions. The set up allowed the doctor to work on the subject's appropriate leg while the nurse worked on the contralateral forearm for blood collection.
- The Biopsy Management Section
    - The primary investigator worked in this area. His responsibilities were: to prepare the subject for the resting metabolic measurements; assisting the doctor in the biopsy procedure; and the handling of the tissue specimens after the biopsy procedure.
- The Blood Collection Section
    - The registered nurse worked in this area. The nurses' responsibilities were: to insert the catheter for blood collection; position the heating pad for proper function of the heated hand technique; and collect the 10cc blood samples throughout the experiment.
- The Blood Management Section
    - A paid research assistant worked in this area. Throughout the complete study there were only two assistants that worked this section with one assistant working all but two of the sessions. The responsibilities of this assistant were: to set-up the blood management area; to take the blood sample from the nurse after each blood

sample was taken; to decanter the blood into appropriate tubes for centrifuge; centrifuge the blood the required time; aliquot the resulting plasma volume (approximately 5 cc) as equally as possible into four different 1.5 ml Eppindorf tubes; store those tubes properly for the duration of the session; and prepare the tubes for proper storage post-testing.

- Metabolic Section
  - A paid research assistant worked in this area. One assistant worked this area for all but one session of the study, during which the primary investigator performed this task. The responsibilities of this assistant were: to properly calibrate and manage the metabolic cart for the three time periods required: resting, exercise and recovery.

When the subject was on the gurney, the doctor, nurse and metabolic station assistant worked on different sides of the subject as shown in Figure B-1. During the exercise session (when the subject was on the cycle ergometer) and the first 15-min or recovery (when the subject sat on the chair) the doctor, nurse and assistant worked on different sides of the cycle and chair, similar to their arrangement relative to the gurney. This allowed each person to perform their task without interfering with and disrupting the other areas.

Each of the three areas required supplies unique to that area. To accommodate this, each area had a daily set-up list that was available in that area for the completion of that task. The evening before a test, the research area was set up. This included marking all the required tubes for blood collection and management and the setting out of all the equipment required for each kit. In addition, the gurney and pillow on which the subject laid were washed and covered with a plastic sheet and a cloth-fitted sheet or pillow case. All laundry work was performed by the U of A laundry services of the Physical Education and Recreation building. All sterile material and kits were supplied by the Medical Supplies store of the U of A W.C. McKenzie Hospital .

Day 3 and 4

The total time required for the CON and INT exercise treatments was approximately 4.5 h. Of this time approximately 2.0 h was required to prepare the subject for obtaining resting metabolic, biopsy and blood samples. Approximately 2.5 h was required to obtain the exercise and recovery values.

Resting Session

At 6:00 am, the principle investigator set the lab up for the day's testing activities. Activities included starting up the metabolic cart , getting ice for the blood management section, laying out the kits on the appropriate tables, and making the area ready for the arrival of the subject. At approximately 6:30 the subject arrived at the lab. Subjects were directed to go to the bathroom, empty their bladder, and change into shorts, T-shirt and socks. When the subjects finished, they laid on the gurney in the correct position with the appropriate leg was facing the doctor's station and the appropriate arm was facing the nurse's station. The limbs to be utilized were randomly selected to ensure there was no dominant limb effect. EMLA^{TM} cream was applied to the leg on an approximate 6" by 8" oval area with the mid-point being the outer aspect of the vastus lateralis. On the forearm the cream was applied to the medial aspect of the forearm approximately 10 cm below the elbow. The timing of this application allowed the EMLA^{TM} cream to be on the subject's thigh and forearm for at least 45-min before the doctor and nurse were ready to work on the subject. This is considered the optimal time frame for this cream to reach maximum effect.

The EMLA^{TM} cream reduced the discomfort for the subject. It was important to make the experience as comfortable as possible for the subject, as subjects had to come in for two testing days (Day 3 and 4). If Day 3 was too uncomfortable, the chances of subjects coming back for the second testing day would be reduced, and, for the data to be of any value, subject data from both testing days was required.

Metabolic measurements

After the cream was put on, a heart rate monitor and head-mounted breathing mask (Hans Rudolph Inc., Kansas City, MO) was fitted on the subject. As part of this fitting, a gel seal was placed on the face-piece to reduce the risk of leaks. When the face piece was properly fitted, the subject was instructed to lay on the gurney in a supine position. At this point, only the subject and primary investigator were in the lab. The subject was instructed to lay as quietly as possible but not fall asleep for the next 35-45 minutes. From this point until after resting metabolic measurements were completed the lab was kept as quiet as possible to acquire the most authentic resting values. The lights were kept on as other members of the testing team (doctor, nurse, etc) would be entering the research area in the next 45 minutes and would require lights to prepare their stations.

The subject lay in this position for 10-min during which the metabolic cart was calibrated and readied to collect data. At the 10-min mark the metabolic cart hose was connected to the subject's mask. The subject was left in this position for an additional 5-min at which time metabolic measurements and heart rates were taken for a 20-min period, the final 10-min of which were averaged to provide mean resting values. At the end of the 20-min period, the mask was removed and the subject was allowed to interact with the research team that was now on-scene. However, the subject was instructed to continue to lie as still as possible on the bed. The research team included: the primary investigator, a doctor, a nurse and two lab assistance. At this point, resting metabolic values had been obtained and the doctor and nurse now prepared the subject to obtain resting biopsy and blood samples.

Biopsy sampling

The doctor began his process by liberally applying Benadine to the area previously covered with the EMLA^{TM} cream. This area was surrounded with sterile towels to create a sterile field around the subjects' thigh, thereby reducing the risk of infection. The doctor then proceeded to inject local anesthetic (2% lidocaine without epinephrine) through the epidermis and dermis to the level of the superficial fascia of three pre-marked biopsy sites. These sites were on the lateral aspect of the vastus lateralis. The middle site was positioned on the lateral most aspect of the muscle. From this position, two more sites were selected, each approximately 4-6 cm to the left or right of the middle site, at the same level on the muscle. After ensuring the sites were properly prepared, the doctor used a scalpel blade to cut a small 2-3 cm incisions along the frontal plane for each of the three biopsy spots. The incisions were to the depth just through the superficial fascia. The doctor insured the bleeding was stopped at all three sites and prepared to obtain the resting biopsy from the proximal incision.

The primary investigator, wearing gloves, lifted the autoclave pouch containing one of the biopsy needles and carefully opened the pouch ensuring that at no time was the needle touched by the primary investigator. At this point, the doctor, now double-gloved, took the biopsy needle out of the autoclave pouch. After assembling the biopsy needle, the doctor inserted the needle into the incision to a depth fully embedded in the muscle tissue. The doctor then pulled back slightly on the plunger of the biopsy needle, opening up the window of the needle within the subjects muscle. The suction tip was then carefully inserted into the plunger of the biopsy needle by the doctor, and the doctor indicated to the primary investigator to apply suction. As suction was applied, the doctor pushed the plunger down, cutting the first muscle “clip”. The doctor then repositioned the needle by moving it slightly to the right and a second clip was acquired. In total, three clips were harvested during each biopsy. This process was completed as quickly as possible to reduce subject discomfort. After the last clip was taken, the needle was removed from the subject at which time the doctor handed the needle to the primary researcher.

Once the resting biopsy was taken, the doctor treated the subject’s incision, using stitches to reduce the risk of infection. The subjects thigh area was then covered with a sterile cloth bandage approximately 6” by 8”. This bandage was taped on the subject in such a way that it could be quickly removed for obtaining the exercise biopsy. At this point the doctor had finished preparing the subject for the exercise portion of the testing day.

Biopsy management post-sampling

For the proper collection of muscle biopsies for enzyme analysis, tissue had to be frozen as quickly as possible and remain in a frozen state until the proper analysis could be performed. To ensure this occurred, the following procedure was followed. Prior to the biopsy being taken, a small table was moved to within two feet of the doctor. On this table was placed a heavy plastic cup (for balance) with a Styrofoam cup inside it. In the minute preceding the biopsy, liquid nitrogen was poured into the Styrofoam cup. As the doctor removed the biopsy needle from the subject it was handed quickly to the primary investigator who inserted in directly into the Styrofoam cup of liquid nitrogen. With this process, the biopsy sample was immersed in liquid nitrogen within approximately 1.0 second of being removed from the subject. The needle was kept in liquid nitrogen until the tissue sample was completely frozen. The needle was then lifted out of the liquid nitrogen and slightly warmed by twisting the needle within a cloth in the investigators hand. When warmed slightly the plunger (which contained the muscle biopsy) would rotate in the outer shell of the needle. When this occurred, the plunger was pulled out of the outer shell of the needle and both the shell and the plunger were reinserted in liquid nitrogen to ensure that none of the muscle tissue would thaw in the process. The plunger containing the biopsy was then warmed slightly using the same process. At the same time, a smaller metal rod (pre-frozen in liquid nitrogen) was periodically pushed down through the hollow chamber of the plunger. This rod was constantly reinserted in liquid nitrogen to keep it frozen between attempts to free the biopsy. As this process proceeded, the small plunger eventually pushed the biopsy sample out of the plunger and directly into liquid nitrogen. With this method the biopsy remained in a frozen state as it was removed from the biopsy needle.

The biopsy sample was then visually inspected as it sat in liquid nitrogen to determine its size and quality. If too small (under 70 mg) or composed primarily of blood or adipose tissue, another biopsy was taken, referred to as a second-sample biopsy. Only one second-sample biopsy could be taken from a subject per testing day, allowing for a maximum of four biopsies per day: resting sample, exercise sample, recovery sample and one second-sample. A second-sample biopsy utilized the same incision, therefore another incision was not required.

Those biopsies that were deemed acceptable were removed from the liquid nitrogen with pre-frozen tweezers and placed in a pre-frozen cryo-tube which was immediately immersed in liquid nitrogen in a portable doer. The samples stayed in this doer until the end of the testing day when they were transferred directly into a larger liquid nitrogen container. From these biopsies, two samples, a 40-60 mg sample for AMPK, and a 10-30 mg sample for PDH_a, were chipped off and dissected free of blood and connective tissue under liquid nitrogen. The remaining frozen muscle was freeze-dried, dissected free of blood and connective tissue and stored dry at –80 °C until it was used for glycogen determination.

Blood sampling

The nurse was responsible for obtaining the resting blood sample. To achieve this the nurse inserted an 18-gauge catheter into the anticubital or basilic vein approximately 15-20 cm below the elbow in the area where the EMLA™ cream had been administered. An extension was attached to the catheter that extended past the top of the heating pad used to warm the hand and forearm region in the administration of the heated-hand technique. The heating pad was wrapped around the hand/forearm region of the subject with one end of the pad extending to the knuckles of the hand and the other end extending to a position approximately 10 cm below the elbow. The heating pad was kept on as high a heat-setting as the subject would tolerate. If the subject complained about the pad being too hot, the temperature was turned down to a medium setting for a few minutes then returned to the high setting. No subject found the medium setting intolerable. After approximately 15-min with the heating pad on and the subject having lain relatively still, the nurse drew the resting 10 cc blood sample into a syringe. After drawing blood, adequate saline was injected into the catheter to keep the extension and catheter from clotting between blood samples. At this point the nurse had finished preparing the subject for the exercise portion of the testing day.

Blood management post-sampling

The syringe was immediately given to the research assistant in charge of blood management. Approximately 0.5 ml of whole blood was injected into a 1.5 ml Eppindorf tube, which was immediately placed on ice. This sample was used for lactate analysis. The remainder of the blood was then carefully decanted, to reduce the risk of rupturing the red blood cells, into a heperanized tube and placed quickly on ice. When three blood samples had been acquired, those three tubes plus a forth tube of water (of a weight to equally balance out the centrifuge) were centrifuged at 10,000 g for a period of 10-min. When removed from the centrifuge, the tube contained approximately 5.0 ml of plasma, which was pipetted equally into four pre-chilled and pre-marked 1.5 ml Eppindorf tubes which were kept on ice throughout the day's testing session. At the end of the testing day the Eppindorf tubes were transported a short distance in pre-chilled boxes to a freezer where they were stored at –20 °C.

Exercise Session

After the resting values were taken, the subject was assisted off the bed and onto the exercise bike (SensorMedics 800S, Yorba Linda, CA). The subject was fitted with a Hans Rudolph one-way breathing valve and mouthpiece (Hans Rudolph Inc., Kansas

City, MO) and prepared to perform the exercise phase of either the CON or INT challenge on the cycle ergometer. Stop watches were used by the nurse, primary investigator and the metabolic cart assistant to coordinate the timeframe for obtaining metabolic measurements, blood and biopsy samples. An overview of the timeframe at which these samples were taken in displayed in Figure 4-1. When the testing team was ready to begin the exercise session, a 5-second countdown was used to enable everyone to synchronize stopwatches. This beginning of the exercise session was considered minute-zero.

Blood samples were taken five times during exercise (at minute 7, 10, 22, 25, 37) and six times during recovery (minute 48, 58, 68, 78, 88, 98). Blood samples during exercise were designed to occur at the following points of the INT exercise treatment: at the end of the high intensity period (minute 7) and the end of the low intensity period (minute 10) of the first interval; at the end of the high intensity period (minute 22) and low intensity period (minute 25) of the fourth interval; at the end of the high intensity period of the seventh interval (minute 37). The timing of the blood samples during CON was identical although the same exercise intensity was used throughout CON.

At approximately 45 seconds before the required time for the blood sample, the nurse would draw out approximately 5.0 cc of blood and saline and discard the syringe. This would ensure that the subsequent blood sample was free of saline. At approximately 30 seconds to the required time, the nurse would connect a 10 cc syringe and prepare to draw the blood sample. At 15 seconds the nurse would begin to draw the blood sample and the draw would be completed by the minute mark. This process was utilized to obtain the blood sample as close to the end of the high intensity and low intensity periods of the

INT exercise treatment as possible. This process was used for all blood samples taken, including those in the recovery period.

The CON challenge consisted of an identical warm-up to INT followed by an exercise session at a PO designed to produce 33 min of exercise of equal work to the INT session. The CON work intensity was below the ventilatory threshold (Tvent) for all subjects. The INT challenge consisted of a 5-minute warm-up followed by a 33-min interval session for a total exercise time of 38 minutes. The interval session consisted of seven intervals in total. The first six periods consisted of 2-min at a PO designed to elicit 90% $V0_{2max}$ followed 3-min at a PO designed to elicit 30% $V0_{2max}$. The final interval consisted of 2-min at a PO designed to elicit 90% $V0_{2max}$ followed 1-min at a PO designed to elicit 30% $V0_{2max}$. The purpose of the 1-min duration for the recovery period of the final interval was two-fold. For safety reasons, the subject required a certain amount of time at the recovery intensity before they could safely be moved from the cycle to the chair. Moving the subject to the chair immediately after the high intensity period could have resulted in blood pooling in the legs, and resulting dizziness, etc. occurring with the subject. In addition, we were attempting to equate min-VO_2 values for the first minute of the INT and CON recovery periods. Data from previous research in this lab utilizing similar INT and CON protocols demonstrated that min-VO_2 for the first minute of recovery following CON exercise was not statistically different than min-VO_2 for the first minute of the low intensity period following the seventh high-intensity period. Differences in initial min-VO_2 has been demonstrated to significantly affect EPOC values and therefore, the attempt to match initial minute VO_2 would allow more direct comparison of EPOC values following the CON and INT exercise treatments.

During the final minute of the high-intensity period of the sixth interval, the assistant managing the metabolic cart would provide verbal time markers of 60, 45, 30, 15 seconds and would count down the final 5 seconds of the minute. With 30 seconds left, the assistant managing the blood, stood behind the subject and prepared to steady him during the biopsy procedure. With 15 seconds left the doctor removed the bandage covering the subject's incisions and a chair was placed in front of the cycle ergometer. At the end of the minute, the subject was directed to stop cycling and straighten the prepared leg placing the heel on the chair. The subject was pre-cautioned not to tighten the muscles in this leg but instead to try to keep as relaxed as possible. The subject kept his other leg in the toe clip. With the assistant now supporting the subject, the biopsy was quickly taken from the middle incision. No stitches were put in the middle incision at this time so as to get the subject pedaling and completing the remainder of the exercise program as quickly as possible. The time between when the subject stopped peddling until they resumed was standardized at 1 min 30 seconds. This was composed of approximately 35 seconds to obtain the biopsy and 55 seconds to manage the biopsy site (stopping the bleeding, re-bandaging, taping, etc.). When calculating the work performed during the exercise session, no work was considered performed during this period. As the subject pedaled for the remainder of the low intensity period, work was recorded for the final 1 min 30 seconds. Standardizing the period of time required for the biopsy allowed more accurate matching of work between the INT and CON session.

The exercise biopsy was taken during the sixth interval and not the seventh because of the need to ensure an adequate biopsy. If a biopsy was obtained in the sixth high interval that was less than 70 mg, another exercise biopsy was required. This was then obtained during the seventh interval.

Recovery

Following the CON and INT exercise treatments the subjects began the one-hour recovery period. The first 15-minute period consisted of the subject moving off the cycle to a chair positioned directly beside the bike. During this period metabolic measurements and blood samples were taken. The second 15-min period consisted of the subject removing the metabolic headgear, etc. and cautiously being moved back to the gurney. Once on the gurney, further blood samples and the recovery biopsy was obtained from the distal incision. The biopsy was taken 26-min into recovery. Data from previous research in this lab utilizing a similar INT and CON exercise protocol demonstrated that the RER following INT exercise reached the lowest value at precisely 26-min post-exercise. It was felt this would be the most appropriate and interesting time to sample tissue and analyze metabolic enzymes and metabolites related to substrate utilization. If the biopsy obtained was less than 70 mg, another biopsy was taken as soon as possible. At the end of this 15-min period, the final biopsy had been acquired and the doctor performed final management of the biopsy site.

For the final 30-min of the recovery period only blood samples and metabolic data were obtained. During this period, as during the resting phase at the beginning of the testing day, the subject was directed to remain still and quite and the lab was kept as quiet as possible. At the end of this 30-min period, the test was terminated.

At the conclusion of testing, the subject was allowed to get up off of the gurney and move around. A fruit drink and a muffin were provided for the subject. The subject was monitored for any complications related to the testing day for approximately 30-min, after which time, if no problems existed, the subject left the testing area.

Table B-1: An example of the power output and oxygen consumption values generated from a VO_{2max} test.

P.O. (watts)	Oxygen Consumption (liters/min)
25	1.010
50	1.190
75	1.510
100	1.540
125	1.930
150	2.190
175	2.460
200	2.570
225	2.950
250	3.280
275	3.560
300	3.870
325	4.010

PO = power output,

Table B-2: An example of an INT exercise treatment program with the accompanying high-intensity and low-intensity power outputs.

TIME	TASK	PO	TIME	TASK	PO
1	Warm		20	LOW	85
2	Warm		21	HI	305
3	Warm		22	HI	305
4	Warm		23	LOW	85
5	Warm		24	LOW	85
6	HI	305	25	LOW	85
7	HI	305	26	HI	305
8	LOW	85	27	HI	305
9	LOW	85	28	LOW	85
10	LOW	85	29	LOW	85
11	HI	305	30	LOW	85
12	HI	305	31	HI	305
13	LOW	85	32	HI	305
14	LOW	85	33	Biop	0
15	LOW	85	34	Biop	42.5
16	HI	305	35	LOW	85
17	HI	305	36	HI	305
18	LOW	85	37	HI	305
19	LOW	85	38	LOW	85

Time = minute of the INT program, WARM = warm-up, HI = high intensity work load, LOW = low intensity work load, PO = power output in watts.

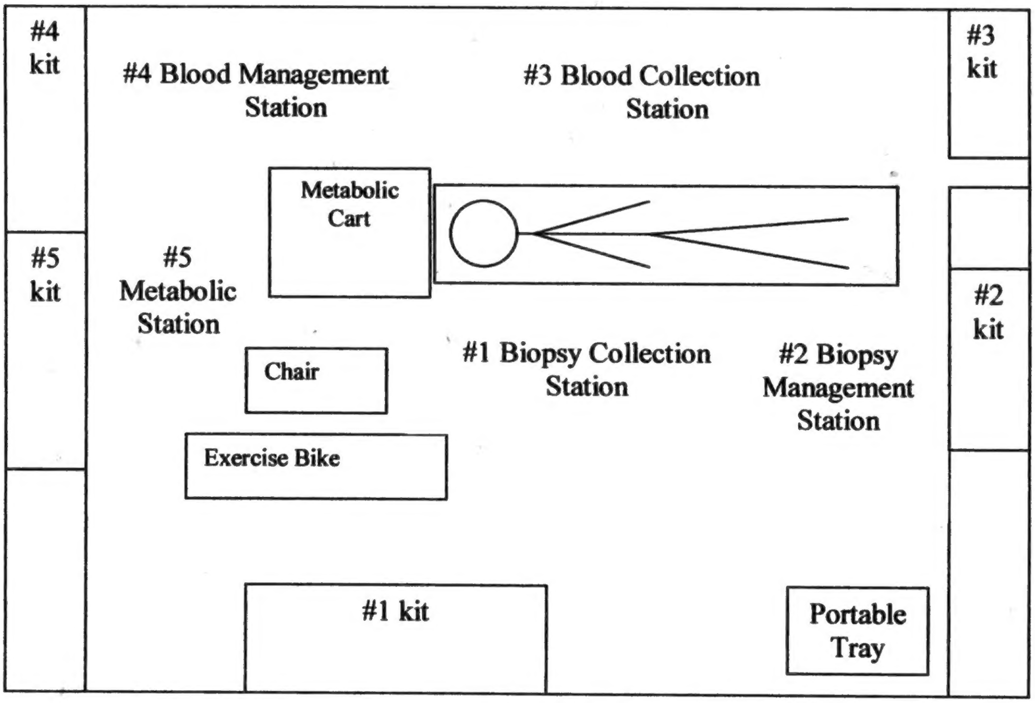
#4 kit
#4 Blood Management Station
#3 Blood Collection Station
#3 kit
Metabolic Cart
#5 kit
#5 Metabolic Station
#2 kit
Chair
#1 Biopsy Collection Station
#2 Biopsy Management Station
Exercise Bike
#1 kit
Portable Tray

Figure B-1: Laboratory setup.

APPENDIX C

EXTENDED METHODOLOGY FOR BLOOD LACTATE ANALYSIS

Introduction

Blood samples were allowed to thaw at room temperature and were subsequently analyzed in duplicate using a Yellow Springs Instrument Model 27 analyzer (Yellow Springs, OH). Samples were analyzed in duplicate and the analyzer was calibrated with lactate standards of known concentration. If the difference between duplicate samples was greater than 0.3 mM, re-calibration was initiated and the samples were re-analyzed. The lactate analyzer was calibrated every 5 samples and in the event of analyzer drift, previous samples were analyzed again. The average value of the duplicates was used for data analysis.

APPENDIX D

EXTENDED METHODOLOGY FOR PLASMA GLUCOSE ANALYSIS

Introduction

Plasma samples were kept frozen at –20 °C until analyzed. A Beckman Glucose Analyzer 2 (Bechman Instruments Inc., Fullerton, C.A.) was used for analysis. The following information on the process involved in the measurement of plasma glucose has been developed from notes accompanying the Beckman Glucose Analyzer 2. This methodology used a process where a precise volume of plasma sample was pipetted into an enzyme reagent contained in an electrode that responds to oxygen concentration. In this process the rate of oxygen consumption, which is directly proportional to the concentration of glucose in the plasma sample, is measured.

$$(1)\ \beta\text{-D-Glucose} + O_2 \xrightarrow[H_2O]{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

Therefore:

(1) Oxygen is consumed at the same rate as glucose reacts to form gluconic acid, therefore, the rate of oxygen consumption was directly proportional to the concentration of glucose present.

The process utilized in operation of the Beckman Glucose Analyzer 2 was as follows:

1. The reagent used in this process was the Beckman Certified Glucose Reagent, which is a special-purpose reagent designed for the Beckman Glucose Analyzer 2.
2. Reagent was stored at 4 °C and warmed to room temperature before being aerated.
3. Reagent aeration occurred with the reagent bottle being shaken vigorously. Afterwards, the bottle cap was removed and the plastic bottle was squeezed until liquid came to the top of the bottle. Then the pressure was released to permit the entry of fresh air into the reagent bottle. This process was repeated twice. The reagent bottle was then installed in the analyzer.
4. The analyzer was primed with reagent by cycling the reagent through five cycle operations by all pumps.
5. The analyzer was then adjusted for oxygen level with a desired value being 450 ± 10. The value we observed was 449.
6. The analyzer was then calibrated with an aqueous standard of 150 mg/dL. After calibration was set at 150 mg/dL, standard was pipetted into system two more times with results being in the range of 147-153 to ensure correct calibration.
7. The analyzer was placed in readiness condition and prepared to operate.
8. The pipetting process utilized was the "forward five" technique which was performed as follows:
    - The pipette utilized was the Beckman's Accustroke Pipette
    - A tip was installed on the pipette with a push-on-and-twist technique which readies the pipette for use
    - The Operating Pushbutton on the top of the pipette was depressed and held down as the pipette tip was placed slightly below the surface of the sample which had been previously thawed and warmed to room temperature
    - The Operating Pushbutton was slowly released, drawing sample into the tip.
    - With the pipette tip being held at approximately 45 degrees, the tip was slowly lowered into and raised from deionized water in a continuous motion. The tip was not stirred in the water nor did it touch the walls of the water container. The tip was lowered just slightly lower in the water than it was into the sample. This process was utilized to remove any excess sample from the exterior of the pipette tip and ensure greater accuracy of measurement.
    - The pipette tip was then firmly inserted into the reaction cup opening of the Analyzer.
    - The Operating Pushbutton was then depressed and released smoothly five times, ending with the Pushbutton held in the depressed position.
    - The pipet was withdrawn from the reaction cup in this position, and with the Pushbutton still in the depressed position, the disposable pipette tip was removed and the Pushbutton released.
9. The glucose concentration value was displayed within approximately 10 seconds and this value was recorded.
10. Duplicates of each sample were performed. If duplicates were more than 3 mg/dL different a third measurement of the sample was performed. If the closest two values were still greater than 3 mg/dL different, a standard calibration was performed.
11. A standard calibration was performed every 10 samples.

12. All samples measurements were performed on the same day.

APPENDIX E

EXTENDED METHODOLOGY FOR GLYCEROL ANALYSIS

Introduction

Plasma samples were kept frozen at –20 °C until analyzed. A Sigma Diagnostics Triglyceride (GPO-Trinder) kit was used for analysis (Sigma Diagnostics, Inc., St. Louis, MO). This process uses a colorimetric determination of glycerol in serum. The following information on the process involved in the measurement of serum glycerol has been developed from methodology notes accompanying the Sigma Diagnostic kit.

(1) $\text{glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{G-1-P} + \text{ADP}$

(2) $\text{G-1-P} + \text{O2} \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$

(3) $\text{H2O2} + \text{4-AAP} + \text{ESPA} \xrightarrow{\text{POD}} \text{Quinoneimine Dye} + H_2O$

Therefore:

(1) Glycerol is phosphorylated by adenosine triphosphate (ATP) forming glycerol-1-phosphate (G-1-P) and ADP in a reaction catalyzed by glycerol kinase (GK)

(2) G-1-P is then oxidized by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2).

(3) A quinoneimine dye is produced by the peroxidase catalyzed coupling of 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl)m-anisidine (ESPA) with H_2O_2 which shows an absorbance maximum at 540 nm. This increase is directly proportional to glycerol concentration of the sample.

The process utilized in operation of the Sigma Diagnostics Triglyceride (GPO-Trinder) kit was as follows:

Preparation of reagent solutions

1. Only Reagent A was used from this kit for the analysis of glycerol.
   - Reagent A contained:
     - ATP, magnesium salt, 4-AAP, ESPA, GK, GPO, POD, buffer and non-reactive stabilizers and fillers
   - Reagent A was reconstituted with deionized water and immediately mixed several times by inversion
2. The reagent was used on the day it was developed and was not exposed to direct sunlight during use.
3. 96-well plates were used for the measurement of standards and samples.
4. A serial dilution method was used to develop the standard curve. In total, seven dilutions of the 250.00 ml/dl standard were developed in 1.5 ml eppindorf tubes. These concentrations were: 250.00, 125.00, 62.50, 31.25, 15.63, 7.81, 3.91 ml/dl mmol/liter. Ten *u*l of each standard concentration was added to the appropriate wells on the 96-well plate. In addition, blank values were prepared using two wells containing 10 *u*l of distilled water
5. 10 *u*l of each sample was added (in duplicate) to each designated well on the plate.
6. Therefore, each 96-well plate contained:
   - duplicates of all seven concentrations of the standard,
   - two wells of distilled water (blanks),
   - 24 wells dedicated to the subjects CON samples (12 samples in duplicate),
   - 24 wells dedicated to the subjects INT samples (12 samples in duplicate).
   - The other wells of the plate were left empty. In this way all of one subject's INT and CON glycerol values were developed on the same plate with the required standards and blanks.
7. 200 *u*l of Reagent A was added to each well and the plate was incubated for 15-min at 37 °C.
8. The plate was read at 540 nm by a Molecular Devices SpectraMax 190 (Sunnyvale, CA) spectrophotometer and generated glycerol values and R^2 values were recorded.
9. Samples with a coefficient of variance greater than 20% were analyzed again. All R^2 values were greater than 0.980

APPENDIX F

EXTENDED METHODOLOGY OF NEFA ANALYSIS

Introduction

Plasma samples were kept frozen at –20 °C until analyzed. A Wako NEFA C kit (Wako Chemicals USA, Inc., Richmond, VA) was used for analysis. This process uses a colorimetric determination of NEFA in serum.

The following information of the process involved in the measurement of serum NEFA has been developed from methodology notes accompanying the Wako NEFA C kit. The process results in NEFA being converted to their copper salts which are extracted into an organic solvent. The salts are then complexed with a dye for purposes of colorimetric measurement. This method relies upon the acylation of coenzyme A (CoA) by the NEFA in the presence of added acyl-CoA synthase (ACS). The acyl-CoA thus producted is oxidized by added acyl-CoA oxidase (ACOD) with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of perodidase (POD) permits the oxidative condensation of 3-metyhl-N-ethyl-N-(B-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple colored adduct which can be measured colorimetrically at 550 nm. The formula is as follows:

$$\text{(1) NEFA + ATP + CoA} \xrightarrow{\text{ACS}} \text{Acyl-CoA + AMP + PPi}$$

$$\text{(2) Acyl-CoA + O2} \xrightarrow{\text{ACOD}} \text{2,3-trans-Enoyl-CoA} + H_2O_2$$

$$\text{(3) } H_2O_2 + \text{4-AAP} \xrightarrow{\text{POD}} \text{MEHA}$$

Therefore:

(1) NEFA in serum, when treated with acyl-CoA synthetase (ACS) in the presence of ATP, magnesium cations and CoA, form the thiol esters of CoA known as acyl-CoA and AMP and pyrophosphate.

(2) Acyl-CoA is oxidized by added acyl-CoA oxidase (ACOD) to produce hydrogen peroxide

(3) Hydrogen peroxide in the presence of peroxidase (POD) allows the oxidative condensation of MEHA with 4-aminoantipyrine to form a purple colored adduct with an absorption maximum at 492 nm

Ascorbic acid (vitamin C) must be removed from all samples due to its role as an antioxidant and reactibility with hydrogen peroxide. Therefore, ascorbate oxidase (AOD) is added to the reaction mix at the outset to remove all ascorbic acid. The process utilized in operation of the Wako NEFA C kit was as follows:

Preparation of reagent solutions

1. Each kit contained the following: 6 bottles of Reagent A powder, 1 bottle of Solvent A, 6 bottles of Reagent B powder, 1 bottle of Solvent B, 1 bottle of 1 mmol/l oleic acid standard.
    - Color Reagent A contains:
        - ACS, AOD, CoA, ATP, 4-aminoantipyrine in dry form
    - Solvent A contains:
        - A aqueous solution with phosphate buffer, magnesium chloride surfactant, and stabilizers.
    - Color Reagent B contains:
        - ACOD, POD in dry form
    - Solvent B contains:
        - MEHA and surfactant in acquous form.
2. All color reagent solutions were used on the day they were developed and were not exposed to direct sunlight during use.
3. 96-well plates were used for the measurement of standards and samples.
4. A serial dilution method was used to develop the standard curve. In total, seven dilutions of the 1.0 mmol/liter standard were developed in 1.5 ml eppindorf tubes. These concentrations were: 1.000, 0.500, 0.250, 0.125, 0.063, 0.031, 0.016

mmol/liter. Ten *ul* of each standard concentration was added to the appropriate wells on the 96-well plate. In addition, blank values were prepared using two wells containing 10 *ul* of distilled water .

5. 10 *ul* of each sample was added (in duplicate) to each designated well on the plate.
6. Therefore, each 96-well plate contained:
   - duplicates of all seven concentrations of the standard,
   - two wells of distilled water (blanks),
   - 24 wells dedicated to the subjects CON samples (12 samples in duplicate),
   - 24 wells dedicated to the subjects INT samples (12 samples in duplicate).
   - The other wells of the plate were left empty. In this way all of one subject's CON and INT NEFA values were developed on the same plate with the required standards and blanks.
7. 80 *ul* of Reagent A was added to each well and the plate was incubated for 10-min at 37 °C.
8. 160 *ul* of Reagent B was added to each well and the plate was incubated for 10-min at 37 °C
9. The plate was read at 492 nm by a Molecular Devices SpectraMax 190 (Sunnyvale, CA) spectrophotometer and generated NEFA values and R^2 values were recorded.
10. Samples with a coefficient of variance greater than 20% were analyzed again. All R^2 values were greater than 0.980

APPENDIX G

EXTENDED METHODOLOGY FOR THE PDH$_a$ ASSAY

Introduction

Determination of PDH$_a$ activity level involved the following processes: analysis of muscle tissue samples to determine Acetyl-CoA levels; conversion of Acetyl-CoA values into PDH$_a$ activity level; determination of protein content of muscle tissue homogenates; correction of PDH$_a$ activity levels to standardized protein content.

Analysis of tissue samples to determine Acetyl-CoA levels

Two primary solutions were required for the analysis: PDH Assay Buffer and PDH$_a$ Homogenizing buffer (HB1). The required reagents and their concentrations for these solutions are listed in Table 1 (Assay Buffer) and Table 2 (HB1). The PDH analysis was a two-stage analysis with Stage I resulting in the production of Acetyl-CoA. Stage II involved the measurement of the Acetyl-CoA, the values of which were used for the calculation of PDH$_a$ activity.

Stage I Materials and Procedure

Materials required for Stage I were:

- Heating block with thermometer
- Vortex
- Timer
- Homogenizers
- Two buckets of ice
- Two positive placement pipettes with tips
- Marked tubes. For every tissue sample you will need:
  - 3 marked 1.5 Eppindorf tubes (series 1 tubes marked as follows: 1-1, 1-2, 1-3)
  - 9 marked 1.0 small tubes (series 2 tubes marked as follows: 1-1-1, 1-2-3, 1-1-3, 1-2-1, 1-2-2, 1-2-3, etc.)
- Small (25-50 ml) Pyrex container
- Regular pipette and tips

  - From 30 µl, 720 µl, to 1.5 ml
  - Yellow and blue tips
- Gloves
- Tissue samples in liquid nitrogen
- 1.5 Eppindorf tube holder
- 4 series-2 tube holders
- Styrofoam cups
- Tweezers
- Porcelain Bowl
- Required Solutions
  - 0.5 M PCA
  - 2.0 KHCO3
  - 39 mM NAD
  - 13mM CoASH
  - 13mM TPP
  - 26mM Pyruvate
  - HB1
  - PDH Assay Buffer

The following procedure was followed for the Stage I analysis.

1. Turn on block heater to 37°C
2. Aliquot 40 µl of 0.5M PCA into each series2 tubes
3. Pipette into each series 1 tube – then place in heating block to warm (at least 3 min)

720 µl	PDH Assay Buffer
80 µl	NAD
80 µl	CoASH
80 µl	TPP

4. Place pyruvate into heating block to keep warm
5. Place 1.5 ml Eppindorf tube of dH_2O into heating block to keep warm
6. Weigh and homogenize tissue samples in pre-chilled homogenizers
   - Place small plastic container filled with ice containing pre-chilled homogenizer on scale
   - Add 100 µl of HB1 to homogenizer
   - Tare
   - Pour tissue sample from Cryo-tube to Styrofoam cup filled with liquid nitrogen, use pre-frozen tweezes to move tissue sample into homogenizer
   - Count to 5 sec
   - Record weight of tissue sample
   - Homogenize sample using glass plunger to gently disintegrate the tissue sample
   - Add extra HB1 for a final ratio of 1mg of muscle tissue /30µl of HB1. For example if your tissue sample weighed 5 mg then the final required volume of

HB1 would be [5 x 30] 150 µl. As 100 of HB1 was added before homogenization, 50 µl should be added at this point to achieve the proper ratio.
  - Transport homogenate with glass pipette to pre-chilled 1.5 Eppindorf tube and place tube on ice.

7. Mix homogenate completely by vortexing and then drawing up and ejecting it from the positive displacement pipette (check to ensure no connective tissue in sample)
8. Add 30 µl of homogenate to FIRST (1-1), and only the first, pre-heated series-1 tube. Mix well by drawing up and ejecting from the positive displacement pipette several times
9. Start the reaction by adding 30 µl of pyruvate (26mM) to the first series-1 tube. Vortex and start the clock immediately
10. After exactly 1-min withdraw 200 µl of the mixture and place in series-2 tubes (containing 40 µl PCA). Vortex and allow to sit on ice until present trial is complete
11. Repeat step #8 at 2 min and 3 min
12. Repeat steps so that the procedure is run in duplicate in series-1 tube #2 for 1,2, and 3 min
13. Run a blank for each homogenate by repeating steps #6 to #9 except adding 30 µl of Millipore ddH_2O at step #9 instead of pyruvate
14. Neutralize the samples by adding 12 µl of 1.0 M K_2CO_3 (Potassium Carbonate) to each series-2 tube being sure to allow 5-min to pass before the blanks are neutralized
15. Freeze neutralized extracts until analysis of acetyl CoA (-80°F)

Stage II Materials and Procedure

Acetyl-CoA standards were developed as follows.

- 500 µmol/l Acetyl-CoA stock was developed by adding 4 mg Na-salt to 10 ml ddH_20
- 5 µmol/l Acetyl-CoA standard was developed by adding 500 µl of 500 µmol/l stock to 50 ml of ddh20
- 1 µmol/l Acetyl-CoA standard was developed by adding 100 µl of 500 µmol/l stock to 50 ml of ddh20

Materials required for Stage II were:

- Stage I samples
- Acetyl-CoA Spreadsheet.
- Centrifuge (set at 4°C) with 1.5 ml Eppindorf tray
- Vortex
- White 5 ml tube container (holds series-3 tubes)
- A number of 1.5 ml Eppindorf tubes
- 10-20 ml tubes for Oxyglutaric acid
- Marked 5 ml tubes for the development of the standard curve you need:
  - 14 x 5 ml tubes with two tubes marked for each required concentration of standard which are listed in Table 3.
- Marked 5 ml tubes for every sample to be analyzed

  - 18 x 5 ml tubes for each sample with two tubes marked for each of nine series-2 tubes that exist for each sample
- Two buckets of ice
- Regular pipette and tips
  - Pipettes for: 2 µl, 20 µl, 30 µl and 500 µl
  - Yellow and blue tips
- Gloves
- Timer
- DH2O
- Scintillation Vials
- Required Solutions
  - 0.5 mol/L, pH 7.4 HEPES
  - 11 mmol/l EDTA, pH 7.4
  - 60 mmol/l EDTA, pH 7.4
  - 6% PCA (1 mol/l)
  - 30 mmol/l NEM
  - 178 mmol/l L-glutamic acid, K-glutamate
  - $CuSO_4$: K-acetate mixture
    - 1 mmol/l $CuSO_4$ is made and frozen in 2 ml portions. 400 mmol/l K-acetate pH 4.8 is prepared and stored in fridge. The solutions are combined as follows: 15 µl $CuSO_4$ x n, plus 15 µl K-acetate x n, plus 10 µl ddH_2O x n and stored frozen in 2 ml portions.
  - One of the 5 umol/l Acetyl-Coa Standard (One of the 1 umol/l Acetyl-Coa Standard
  - 40 umol/l L-U-^{14}C aspartate
  - Citrate Synthase (CS) made according to Acetyl-CoA spreadsheet
  - GOT made according to Acetyl-CoA spreadsheet
  - 10 ml 2-oxyglutarate made according to Acetyl-CoA spreadsheet
  - Dithiotretol (DTT) made according to Acetyl-CoA spreadsheet
  - Resin Solution made by adding 18 g of Resin (Dowex 50W) to 45 ml ddH_2O
  - Transamination mix made according to Acetyl-CoA spreadsheet

The following procedure was followed for Stage II analysis.

Steps common to Standard Curve and Samples
1. Gather materials
2. Label all 5 ml tubes and duplicate series-2 tubes
3. Develop solutions that are required to be made "the day of" (CS, GOT, 2-oxyglutarate, DTT)

For development of the Standard Curve
1. Add Standard Solutions and ddH_2O to pre-marked 5 ml tubes according to Table 3 then follow common steps.

For the measurement of Acetyl-CoA in samples

1. Put proper centrifuge tray to hold series-2 tubes in fridge to cool 30-min before use.
2. Set centrifuge to 4°C 15-min before use.
3. Place duplicate set of sample series-2 tubes (the ones the supernatant will be transferred into) in fridge to chill.
4. Thaw samples in ice to be measured that day, vortex samples then centrifuge at 12,000g, 4 °C for 3-min, transfer (pipette) supernatant (approx. 200 μl) to pre-chilled, identically marked series 2 tubes.
5. Pipette sample and dH_2O into pre-marked 5 ml tubes as follows. For the rest and recovery protocol samples use 20 μl of sample and 115 μl of ddH_2O (total volume 135 μl). For exercise protocol samples use 10 μl of sample and 125 μl of ddH_2O (total volume 135 μl).

Steps common to Standard Curve and Samples

4. Combine CuSO4: K-acetate mix and DTT (10:1 ratio) e.g. 800 μl CuSO4: K-acetate and 80 μl DTT
5. Add 20 μl CuSO4: K-acetate, DTT mixture to all 5ml tubes, vortex, incubate for 30 min at room temperature (RT)
6. Cool on ice for 10-min
7. In Hot Room, prepare ^{14}C-OAA at this time. Start at the 10-min mark of the 30-min incubation as this label is only stable for one hour.
8. Add 20 μl of 60 mM EDTA, vortex 5-min RT
9. Add 30 μl 30mM NEM, vortex 5-min RT
10. In Hot Room, Add 20 μl of ^{14}C-OAA and 10 μl CS, vortex and incubate 20-min RT
11. Prepare transamination mix
12. In Hot Room, add 30 μl transamination mix, vortex, incubate at RT for 20-min.
13. Make up Resin
14. In Hot Room, add 1 ml of Resin, cap tubes and invert for 2 min
15. Centrifuge at 3000 rpm for 2-min at RT
16. In Hot Room, pre-fill scintillation vials with 5 ml scintillation fluid (ACS NACS104)
17. In Hot Room, add 500 μl of supernatant into scintillation vials
18. Place vials in the beta counter and count for 5-min or 10,000 cpm. This produces a printout with counts per minute (CPM) for each sample.

Calculation of PDH_a values for CON and INT

The process for the conversion of the CPM generated from Stage II analysis to PDH_a activity levels was as follows.

1. Recording of CPM count
2. Determination of Acetyl-CoA (pmol)
    - The first step was the use of the CPM data generated from the standards in Stage II for the development of a regression formula

- Using this regression formula, the CPM's that were developed for the various series of sample tubes (1-1,1-2,1-3, 2-1,2-2,2-3,3-1,3-2,3-3) were inserted into the regression formula producing the corresponding Acetyl-CoA numbers (pmol)

3. Filling in the Time column.
   - The time was either 1-min, 2-min or 3-min referring to the time period at which the samples was drawn off in stage.
4. Determining the amount of muscle tissue used
   - The first step was to determine the concentration of muscle tissue in the muscle homogenate solution. This is determined by calculating the amount of muscle to the solution it was homogenized in (HB1).
   - The relationship was 1 mg of muscle tissue in 30 μl of HB1 (a factor of 1/30). Therefore, if you had a muscle sample with a mass of 5 mg it would be added to a total of 150 μl of HB1. Therefore, after homogenation, the final volume was 150 μl of HB1 and 5 μl of homogenate or 155 μl or .155 ml. This resulted in a concentration of muscle tissue in mg/ml of (5mg/.155ml) or 32.258 mg/ml. If these values were converted this to μg/μl we would get (the same relationship) 32.258 μg/μl.
   - The next step is to determine the amount of solution that the muscle homogenate was added too. Table 4 displays the various stocks and volumes involved. There was some differences with this solution relative to past methodologies as some stocks should have been added in different volumes. Dr. Ted Putman reviewed our process and stated that all volumes meet the proper criteria. The final volume was 1020 μl.
   - Therefore, if 30 μl of homogenate added to a volume brought that volume to 1020 μl, then the homogenate is diluted 34 times (1020/30 = 34). This would result in the new concentration of muscle tissue in solution being (32.258 μg/μl divided by 34) 0.949 μg/μl
   - 200 μl of this concentration was drawn out at 1,2 and 3-min intervals in Stage I and added to two other solutions. Those solutions were 40 μl of PCA and 12 μl of K_2CO_3.
   - Therefore the muscle tissue present in 200 μl of solution (200 times 0.949 μg/μl) was further diluted by the addition of these other 52 μl of solution.
   - As the solution was increased by a factor of 1.26 (252/200) then the concentration of muscle tissue (32.258 μg/μl) must be divided by the same value. Therefore the concentration of muscle tissue at the end of Stage I is (0.949 / 1.26) is 0.75 μg/μl
   - Therefore, the amount of muscle tissue analyzed in Stage II was based upon this value in the following ways:
     - For Resting samples and Post-Exercise samples (for which we used 20 μl of the solution generated from Stage I) 20 μl times 7.5 μg/μl produced a resulting amount of muscle tissue of 15 μg.
     - For Exercise samples (for which we used 10 μl of the solution generated from Stage I, 10 μl times 7.5 μg/μl produces a resulting amount of muscle tissue of 7.5 μg.

5. Linear regression
    - A regression formula was calculated using the three Acetyl-CoA values (min 1,2,3) for each sample. From this regression formula the slope of the line was used in further calculations.
6. PDH_a (mmol/min/kg ww)
    - To calculate this value, we divided the slope of the regression line by the value representing the amount of tissue.
    - For example, if the slope of the line was 5.58191 and the tissue amount was 14.89, then the resulting PDH_a value was 0.37 mmol/min/kg wet wt.
    - This process was performed for all three sections of CPM data for each biopsy sample. Sample one and two which were duplicates of each other and represented Acetyl-CoA values when pyruvate was utilized as the trigger in the reaction, and the third section of CPM data which represented Acetyl-CoA values when ddH_2O was substituted for pyruvate.
7. PDH_a correction (mmol/min/kg ww)
    - The correction value was the PDH_a value for the third control section utilizing ddH_2O rather than pyruvate. This value was subtracted from the two PDH_a values generated with the use of pyruvate.
8. Mean

- The mean was the average of the two PDH_a values from step 7.

9. SD

- This was the SD of the results of the two PDH_a values from step 7.

10. CV%

- This was the coefficient of variation using the data from step 8 and 9 (SD/mean * 100)

At this point mean and SE PDH_a values had been calculated for all tissue samples. The next step was to determine the protein content for all tissue sample homogenates and correct all PDH_a values to a standard protein content.

Protein Assay

Protein analysis was performed on all sample homogenates utilized in the PDH analysis. The process used was the Bio-Rad Protein Assay based on the method of Branford. It is a simple and accurate procedure for determining concentration of solubilized proteins. It involved the addition of an acidic dye to protein solution and subsequent measurement at 595 nm with a spectrophotometer. Comparison to a standard curve provided a relative measurement of protein concentration. Based upon these values,

PDH_a results from the previous section would then be corrected to a specific protein concentration. The basic principle of this dye-binding assay was that a differential color change of the dye occurs in response to various concentrations of protein. The absorbence maximum of an acidic solution of the dye shifts from 465 nm to 595 nm when binding to protein occurs.

The procedure was as follows.

1. Removed homogenate samples from liquid nitrogen and placed on ice to thaw.
2. Removed BSA 1mg/ml sample from the freezer (in 500 ul tubes) and placed on ice to thaw.
3. To reconstitute the lyophilized bovine bovine serum albumin (BSA) standards, we added 20 ml of deionized water and mixed until dissolved. This step was not necessary in this laboratory as the BSA samples had already been reconstituted.
4. Prepared dye reagent by diluting one part dye reagent concentration with four parts distilled deionized (DDI) water. Filtered the dye solution through Whatman #1 filter to remove particles. This diluted reagent may have been used for approximately two weeks if kept at room temperature.
    - Using a 250 ml measuring vial, fill with 160 ml of MilliQ water (ddH20)
    - Using a 50 ml measuring vial, fill with 40 ml of Bio-Rad Protein Assay Solution (PAS)
    - Pour the PAS into the ddH2O, and invert the 250 ml vial numerous times until solution was sufficiently mixed
    - Double fold a #2 Whatman filter paper and position in a funnel placed in a receptacle.
    - Slowly poured the PAS into the filter paper until all the solution had been filtered.
    - Marked the PAS receptacle with the date of the procedure. This solution could have been used for the next 14 days.
5. Prepare cuvettes for development of the Standard Curve
    - Prepared and labeled a series of 16 cuvettes (1 zero and 15 triplicates of different BSA volumes)
        - One zero cuvette = 1 ml of dye solution (marked 0)
        - Three 2 μl cuvettes = 2 μl BSA solution and 998 μl dye solution (marked 2-1, 2-2, 2-3) and vortexed each cuvette immediately after adding BSA
        - Three 4 μl cuvettes = 4 μl BSA solution and 996 μl dye solution (marked 4-1, 4-2, 4-3)
        - Three 6 μl cuvettes = 6 μl BSA solution and 994 μl dye solution (marked 6-1, 6-2, 6-3)
        - Three 8 μl cuvettes = 8 μl BSA solution and 992 μl dye solution (marked 8-1, 8-2, 8-3)

    - Three 10 μl cuvettes = 10 μl BSA solution and 990 μl dye solution (marked 10-1, 10-2, 10-3)
    - BSA solutions were in dye (in cuvette) for at least 5 min (but not more than 1-h) before they were read.

6. Produce Standard Curve
    - Set wavelength on the Spectrophotometer (Pharmacia BioTech Ultra Spec 3000 UV/Visible Spectrophotometer) to 595 nm
    - Run Standards and produced standard curve with a range from 2.00 to 10.00 μg/ml.
7. Run Samples
    - One zero cuvette = 1 ml of dye solution (marked 0)
    - Triplets for each sample, containing 1.0 μl of designated homogenate and 999 μl of dye solution
    - After running the samples, the standard deviation (SD) was between 0.2 and 0.3 or the sample was redone.
    - Sample values were generated in μg/ml.
8. Correct PDH$_a$ values to standardized protein content.
    - All PDH$_a$ sample values were corrected to a standardized protein content of 10.0 μg/ml. For example, if the protein content of a sample was 8.86 μg/ml then the required adjustment to the tissue amount (from the PDH$_a$ calculation) was performed as follows: 8.86 μg/ml/ 10.00 μg/ml = Xμg / 7.5 μg (tissue amount for exercise samples). The resulting value would be 6.64 μg. Therefore 6.64 was substituted for 7.5 as the tissue amount in the calculation of PDH$_a$.
    - All PDH$_a$ values were corrected in this way

Table G-1. Reagents and concentrations for the PDH Assay Buffer

Reagent	[] mM	FW (g/mol)	Predicted g/200ml	Actual g/200 ml
Tris Base	1.97 / 5 =	121.1	0.394	0.398
Tris Acid	5.32 / 5 =	157.6	1.064	1.072
EDTA	0.72	372.24	0.0536	0.0528
MgCl2	1.44	95.21	0.02742	0.02722
DTT	1.44	154.30	0.04444	0.04464

Tris Base (1.97 g/l) and Tris Acid (5.32 g/l) values acquired from Products for Life Science Research 2003-2004 (Sigma-Aldrich Canada Ltd., Oakville Ontario) page 990 for pH 7.80 and temperature 25°C. Final solution adjusted to a pH of 7.80. [] = the concentration of the reagent required., FW = formula weight, Predicted g/200ml = the predicted mass required for the final 200 ml solution, Actual g/200 ml = the actual mass of each reagent included in the final 200 ml solution.

Table G-2. Reagents and concentrations for PDH_a Homogenizing Buffer

Reagent	[] mM	FW (g/mol)	Predicted g/200ml	Actual g/200 ml
Sucrose	200	342.30	13.692	13.695
KCl	50	74.55	0.7455	0.7451
MgCl2	5	95.21	0.0952	0.0952
EGTA	5	380.40	0.3804	0.3804
Tris HCl	50	157.60	1.5760	1.5763
Na F	50	41.99	0.4199	0.4194
DCA	5	99%	83.1 µl	83.1 µl
DTT	1	154.3	0.0309	0.0303
Triton X-100	0.1% (V:V)	0.10 ml	0.20 ml	0.20ml

Final solution adjusted to a pH of 7.80. [] = the concentration of the reagent required., FW = formula weight, Predicted g/200ml = the predicted mass required for the final 200 ml solution, Actual g/200 ml = the actual mass of each reagent included in the final 200 ml solution.

Table G-3. Combination of standard volumes and dH2) volumes required for development of standard curve for Stage II PDHa analysis.

Tube	Standard Concentration (mmol/l)	Volume (µl)	DH2O volume (µl)
0	0	0	135
5	1	5	130
12.5	1	12.5	122.5
25	5	5	130
50	5	10	125
75	5	15	120
100	5	20	115

Table G-4. Stocks and volumes comprising the final solution for calculation of tissue amount.

Stock	mM	Volume (μl)	Final mM
Assay Buffer	N/A	720	N/A
NAD	39 mM	80	3
TPP	13 mM	80	1
CoASH	13 mM	80	1
Pyruvate	26 mM	30	0.8
Homogenate	N/A	30	N/A
Total		**1020**	

APPENDIX H

EXTENDED METHODOLOGY FOR AMPK ASSAY

Advanced preparation AMPK analysis

- Homogenization Buffer (HB)
- Re-suspension Buffer (RB) (Table 2)
- Assay Buffer (AB)
- AMARA
- Mg Cl_2
- Pre labeled:
  - 0.5 ml tubes for homogenates
  - 1.5 ml tubes for supernatant #1 (s/n #1)
  - 1.5 ml tubes for s/n #2
  - 1.5 ml tubes for s/n #3 (or discard)
- Tables or spreadsheet - This spreadsheet with calculate the amount of radio active material you must use to get the desired effect. The radio active material degenerates at a constant rate, therefore after 10 days you may need 2x the radioactive material to get the same effect as the first day.

A. Homogenization of Muscle Powder

1. Prepare homogenization buffer (HB) and re-suspension buffer (RB)

Reagents required for Homogenizing Buffer

Reagent	[] mM	FW (g/mol)	Predicted g/100ml
Tris Base	50	121.1	0.6057
EDTA	1.0	372.24	0.03722
EGTA	1.0	380.4	0.03804
NaF	50	41.99	0.2100
Na pyrophosphate	50	446.06	0.2230

Glycerol	10%
Triton X-100	0.1%

Reagents required for Re-suspension Buffer

Reagent	[] mM	FW (g/mol)	Predicted g/100ml
Tris Base	100	121.1	1.2114
EDTA	1.0	372.24	0.03722
EGTA	1.0	380.4	0.03804
NaF	50	41.99	0.2100
Na pyrophosphate	50	446.06	0.2230

- Glycerol 10%

- These solutions (HB and RB) were pH to 7.5 then adjusted to 90 ml, then glycerol was added to bring the volume to 100 ml. Solutions were then aliquoted into 10 ml portions and stored at 4° C.
- Just prior to use, the following were added for each 10 ml:
  - 100 μl of fresh 100 mM DTT (ICN 856126 3525C).
  - 0.0154 g/ml dH2O
  - One Complete Mini tablet (Roche 1836153 92534200)
- All reagents were kept on ice during use.

2. Tissue samples to be analyzed were removed from the liquid nitrogen storage tank and placed in a smaller Doer containing liquid nitrogen.
3. 1.5 ml microfuge tubes were pre-labeled and kept on ice. These tubes received the homogenizing samples.
4. The 1.0 ml glass homogenizers were cooled on ice and a pair of tweezers were put into liquid nitrogen.
5. Prepared a beaker with ice with a depression to accommodate one glass homogenizer and place the unit on the analytical balance.
6. Placed the homogenizer in the depression and when appropriate Tare the balance.
7. Using pre-cooled tweezers, transferred the tissue sample from its individualized plastic container (immersed in liquid nitrogen) to the homogenizer and recorded the weight.
8. Immediately added four (4) volumes of ice-cold HB, inserted the plunger and homogenized the tissue sample. For example, if tissue sample weighed 50 mg , 200 μl of HB was added.
9. Transferred the homogenate to pre-labeled, pre-chilled 1.5 ml Eppindorf microfuge tubes

B. PEG Purification of Homogenized Muscle

1. Centrifuged the tubes at 14,000 x G for 20-min at 4° C.
2. Prepared freshly made 25% PEG 6000 in dH2O (5.0 g bringing the final volume to 20 ml) and kept on ice.
3. Prepared freshly made 6% PEG 6000 in RB (0.3 g bringing the final volume to 5.0 ml) and kept on ice.
4. Transferred the supernatant (s/n #1) to a microfuge tube and recorded the volume in a table. Multiplied the s/n volume by 1/9 to determine the volume of 25% PEG added to the s/n to yield 2.5% PEG, then added.
5. Inverted on mixer for 10-min at 4 °C.
6. Centrifuged at 10,000 x G for 10-min at 4 °C.
7. Transferred the supernatant (s/n #2) to a tube and recorded the volume. Multiplied this volume by 3.5/19 to determine the volume of 25% PEG to yield a final PEG concentration of 6% and added
8. Inverted on mixer for 10 min at 4° C.
9. Centrifuged at 10,000 x G for 10-min at 4° C
10. Disposed of the s/n and washed the pellet with 6% PEG in RB buffer. Used a wash volume similar to the first supernatant volume. Re-suspended the pellet by pipetting up and down to loosen the pellet, followed by vortexing.
11. Centrifuged at 10,000 x G for 10-min at 4° C.
12. Removed all the supernatant then re-suspended the pellet in 50 μl RB
13. Removed 3 times 3 μl to determine protein level and exported to a spreadsheet
14. Prepared spreadsheet to determine the portions of sample, RB, and 1% Triton to standardize the samples to 1 mg protein/ml on the day of assay. Therefore, the samples were frozen and thawed only once
15. Stored samples at –80 °C until day of assay.

C. Preparation for AMPK Assay

1. Added freshly prepared DTT and CompleteMini to RB as above and kept on ice.
2. Thawed samples on ice and standardized to 1 mg protein/ml RB with 0.1% Triton.
3. Located AMPK Assay Calculation Document and entered sample number and days to decay. This file determined tube number, master mix volumes, ^{32}P-ATP/ATP volumes, etc. Printed worksheet. Noted that each sample was tested under three conditions in duplicate, requiring six (6) tubes per sample.
4. Located assay buffer (stored at –80 °C) and placed on ice to thaw.

Reagents required for Assay Buffer (AB)

Component	Initial Concentration	Mass/10 ml	Assay Concentration
HEPES – NaOH	80 mM	0.1906 g	40 mM
NaCl	160 mM	0.0935 g	80 mM
Glycerol	16%	1.6 ml	8%
EDTA	1.6 mM	0.00596	0.8 mM

- Brought the pH of the Assay Buffer to 7.0 and aliquoted and stored at –80° C.

5. Located 2.5 mM AMARA (stored at –80° C) and placed on ice to thaw. (0.03855 g/10 ml)
6. located 100 mM $MgCl_2$ (stored at 4° C) and placed on ice to thaw. ACS 474 124666 FW 203.3. Weighed out 0.2033 g/10ml dH2O.
7. Prepared fresh (with added MilliQ) and keep on ice.
    - 5.0 mM ATP Sigma
    - 2.5 mM AMP Sigma
    - 100 mM DTT 0.0154 g/ml
8. Prepared master mixes according to volumes on Assay Calculation document
9. Alliquoted master mixes volumes into appropriate tubes as shown in table below:

Reagent	++	+-	- -
AB	12.5 μl / tube	12.5 μl / tube	12.5 μl / tube
AMARA	2 μl	2 μl	-
AMP	2 μl	-	-
DTT	0.2 μl	0.2 μl	0.2 μl
DH2O	3.8 μl	5.8 μl	7.8 μl

10. Prepared "hot" ATP mix/tube as follows:

Reagent	Volume (μl)
32P-ATP at 1 ^{mCi}/ 100 ul	0.1
ATP	1.0
$MgCl_2$	1.25
ddH2O	0.15

D. AMPK Assay

1. The following materials were gathered.
    - Gloves
    - Ring
    - 3 x 100 ul tips
    - 3 x 15 ul tips
    - timer
    - prepared tubes
    - calculator
    - tray of numbered paper slips with one bent corner
    - pen/pencil/marker
    - 1 liter acetone
    - dilute phosphoric acid (20 ml + 1980 ml dH2O)

- small tube of dH2O

2. Removed ^{32}P from freezer and let thaw (time approximately 1-h)
3. Placed dilute phosphoric acid on stir plate (40 ml added to 3960 ml dH2O)
4. Turned on the Geiger counter, checked the battery and placed on lowest setting for detection.
5. Turned on the heater and filled the holes ½ full with dH2O with the temperature at 30° C.
6. Set up a station with shields, bench coat, hot plate, samples on ice, reaction tubes, timer, forceps, paper slips, pipettors (2 µl, 2.5 µl, 15 µl), tips.
7. Prepared the hot mix behind the shield as follows:
    - Removed cap
    - Used 100 µl tip to remove inner lid and place tip in cap
    - Removed calculated volume and added to ATP mix
    - Returned 32P to freezer and checked gloves for radioactivity (RA)
    - Added dH2O to hot ATP mix
8. Filled the washing beaker duet with dilute phosphoric acid and placed on stir plate.
9. Assayed on sample at a time for a total of 6 tubes, 2 tubes for each of the three conditions, as follows:
    - Added 2 µl of sample to each tube, didn't mix, and placed in heater.
    - Added 2.5 µl of hot mix to first tube, vortexed, returned to heater and started timer.
    - Started one tube every 30 sec, therefore finished at 2:30
    - Incubated each tube for 5-min
    - Used forceps to pick up appropriate paper slip by folded corner.
    - At 4:45 removed 15 µl from the first tube and applied to paper.
    - At 5:00 dropped into stirring phosphoric acid.
    - Discarded tube
    - Repeated procedure with remaining tubes every 30 sec.
    - Let the papers accumulate in the acid.
10. When all the samples were run, washed in 3-4 changes of acid for 5-min each. First two washes go into RA waste, remainder of the washes were discarded down the sink and flushed with lots of waters. Wash in acetone 2 x 5-min then dried on paper towels.
11. Prepared scintillation vials with 4-5 ml of Ecolite flur per vial. Working in sequential order, dropped in one paper slip per vial, capped and labeled vials, added 2.5 µl to hot mix to specific activity vial, added 15 µl of – mix which had 2 µl dH2O rather than sample.
12. Operated the counter.

APPENDIX I

ENZYME/SUBSTRATE INVESTIGATION SUBJECT DATA FOR BLOOD AND MUSCLE MEASUREMENTS

Table I-1: PDH data for all subjects

subject	INT: rest	INT: ex	INT: recov	CON: rest	CON: ex	CON: recov
1	0.91	2.14	0.10	0.93	1.07	0.22
2	0.54	1.09	0.83	0.56	1.28	0.29
3	0.17	4.29	0.95	0.27	1.31	0.37
4	1.19	2.05	0.11	0.35	1.84	0.34
5	0.30	3.12	0.42	0.84	1.28	1.00
6	0.54	1.06	0.98	0.40	1.96	0.90
7	0.52	6.27	0.22	0.37	2.11	0.52
8	0.20	1.80	0.28	0.15	1.48	0.93
9	0.47	2.71	0.86	0.64	1.05	0.26
mean	0.54	2.73	0.53	0.50	1.49	0.54
SE	0.11	.56	0.12	0.09	0.13	0.11

All values in mmol·kg wet wt. · min^{-1}. INT: rest = subject values from the biopsy taken during the INT resting stage; INT: ex = subject values from the biopsy taken during the INT exercise stage; INT: recov = subject values from the biopsy taken during the INT recovery stage: CON: rest = subject values from the biopsy taken during the CON resting stage; CON: ex = subject values from the biopsy taken during the CON exercise stage; CON: recov = subject values from the biopsy taken during the CON recovery stage; mean = the average of the values; SE = the standard error.

Table I-2. AMPK data for all subjects

subject	CON: rest	CON: ex	CON: recov	INT: rest	INT: ex	INT: recov
1	871	947	1212	1156	1253	1623
2	568	1111	687	753	809	1020
3	1049	1049	1061	1049	2336	1158
4	1026	636	743	1078	699	1322
5	1399	1558	1460	1186	1716	1843
6	784	995	802	1024	1842	1013
7	1014	1054	557	1014	2611	1133
8	686	1026	840	246	900	817
9	1222	613	518	931	2309	1627
mean	958	999	876	937	1608	1284
SE	87	92	104	96	241	115

All values in pmol·min· mg protein. CON: rest = subject values from the biopsy taken during the CON resting stage; CON: ex = subject values from the biopsy taken during the CON exercise stage; CON: recov = subject values from the biopsy taken during the CON recovery stage: INT: rest = subject values from the biopsy taken during the INT resting stage; INT: ex = subject values from the biopsy taken during the INT exercise stage; INT: recov = subject values from the biopsy taken during the INT recovery stage; mean = the average of the values; SE = the standard error.

Table I-3. Muscle glycogen data for all subjects

subject	CON: rest	CON: ex	CON: recov	INT: rest	INT: ex	INT: recov
1	375	171	192	280	158	92
2	501	230	309	532	405	291
3	295	257	262	359	49	87
4	408	404	384	373	262	296
5	207	87	306	134	43	44
6	390	368	312	316	110	415
7	300	252	282	300	139	103
9	316	349	240	351	157	187
mean	349	265	286	331	165	189
SE	31	38	20	29	42	47

All values in mmol·kg dry wt. CON: rest = subject values from the biopsy taken during the CON resting stage; CON: ex = subject values from the biopsy taken during the CON exercise stage; CON: recov = subject values from the biopsy taken during the CON recovery stage: INT: rest = subject values from the biopsy taken during the INT resting stage; INT: ex = subject values from the biopsy taken during the INT exercise stage; INT: recov = subject values from the biopsy taken during the INT recovery stage; mean = the average of the values; SE = the standard error.

Table I-4. Non-esterfied fatty-acids (NEFA) data for all subjects for CON resting and exercise values

subject	CON:rest	CON: min7	CON:min10	CON:min22	CON:min25	CON:min37
1	458	379	320	224	382	456
2	351	383	370	390	452	537
3	544	289	330	293	315	301
4	564	431	378	332	284	423
5	461	496	440	450	381	510
6	336	333	344	333	365	390
7	361	354	393	363	362	463
8	591	398	394	337	396	403
9	706	435	411	420	426	569
mean	486	389	376	349	374	450
SE	42	20	13	23	17	27

All values in $mg \cdot dl^{-1}$. CON: rest = subject values from the sample taken during the CON resting stage; CON:min7 = subject values from the sample taken during the CON exercise stage at minute 7; CON: min10 = subject values from the sample taken during the CON exercise stage at minute 10: CON: min22= subject values from the sample taken during the CON exercise stage at minute 22; CON: min25= subject values from the sample taken during the CON exercise stage at minute 25; CON: min37= subject values from the sample taken during the CON exercise stage at minute 35; mean = the average of the values; SE = the standard error.

Table I-5. Non-esterfied fatty-acids (NEFA) data for all subjects for CON recovery values

subject	CON: min48	CON: min58	CON: min68	CON: min78	CON: min88	CON: min98
1	581	556	549	564	575	576
2	650	732	548	499	597	547
3	509	486	603	585	523	779
4	450	495	619	452	523	538
5	842	775	702	725	809	633
6	538	426	361	366	331	427
7	795	785	670	508	498	747
8	700	670	596	480	502	647
9	643	545	466	375	451	402
mean	634	608	568	506	534	588
SE	43	45	35	37	43	43

All values in $mg \cdot dl^{-1}$. CON:min48 = subject values from the sample taken during the CON recovery stage at minute 48; CON:min58 = subject values from the sample taken during the CON recovery stage at minute 58; CON:min68 = subject values from the sample taken during the CON recovery stage at minute 68; CON:min78 = subject values from the sample taken during the CON recovery stage at minute 78; CON:min88 = subject values from the sample taken during the CON recovery stage at minute 88; CON:min98 = subject values from the sample taken during the CON recovery stage at minute 98; mean = the average of the values; SE = the standard error.

Table I-6. Non-esterfied fatty-acids (NEFA) data for all subjects for INT resting and exercise values

subject	INT:rest	INT: min7	INT:min10	INT:min22	INT:min25	INT:min37
1	305	436	317	310	317	455
2	188	195	186	209	240	236
3	629	440	390	569	606	508
4	293	257	256	282	292	303
5	479	415	413	475	455	427
6	470	420	410	324	372	446
7	418	360	252	242	307	345
8	575	454	501	422	447	433
9	698	601	529	502	484	401
mean	451	398	362	371	391	395
SE	56	39	39	42	39	28

All values in mg·dl^{-1}. INT: rest = subject values from the sample taken during the INT resting stage; INT:min7 = subject values from the sample taken during the INT exercise stage at minute 7; INT: min10 = subject values from the sample taken during the INT exercise stage at minute 10: INT: min22= subject values from the sample taken during the INT exercise stage at minute 22; INT: min25= subject values from the sample taken during the INT exercise stage at minute 25; INT: min37= subject values from the sample taken during the INT exercise stage at minute 35; mean = the average of the values; SE = the standard error.

Table I-7. Non-esterfied fatty-acids (NEFA) data for all subjects for INT recovery values

subject	INT:min48	INT: min58	INT:min68	INT:min78	INT:min88	INT:min98
1	387	349	291	316	365	371
2	249	236	248	236	183	229
3	409	315	259	277	235	281
4	277	231	232	211	240	241
5	585	681	697	617	690	731
6	462	450	485	527	570	491
7	334	339	344	302	299	478
8	438	314	341	271	321	455
9	416	477	465	492	644	814
mean	395	377	374	361	394	455
SE	34	47	50	48	63	69

All values in mg·dl^{-1}. INT:min48 = subject values from the sample taken during the INT recovery stage at minute 48; INT:min58 = subject values from the sample taken during the INT recovery stage at minute 58; INT:min68 = subject values from the sample taken during the INT recovery stage at minute 68; INT:min78 = subject values from the sample taken during the INT recovery stage at minute 78; INT:min88 = subject values from the sample taken during the INT recovery stage at minute 88; INT:min98 = subject values from the sample taken during the INT recovery stage at minute 98; mean = the average of the values; SE = the standard error.

Table I-8. Glycerol data for all subjects for CON resting and exercise values

subject	CON:rest	CON: min7	CON:min10	CON:min22	CON:min25	CON:min37
1	25.3	44.4	47.7	46.7	51.3	60.3
2	21.9	60.6	61.5	78.5	95.1	91.5
3	28.9	46.6	44.5	46.8	57.5	70.8
4	44.8	45.9	49.7	57.9	61.7	70.5
5	39.8	40.3	45.4	58.8	57.1	67.3
6	24.0	33.2	47.1	50.1	44.4	59.1
7	30.4	26.0	30.1	31.1	54.6	62.8
8	44.7	38.9	47.3	47.5	51.7	54.1
9	30.0	29.4	32.7	38.6	44.5	50.7
mean	32.2	40.6	45.1	50.7	57.5	65.2
SE	2.9	3.5	3.1	4.5	5.1	4.0

All values in $mg \cdot dl^{-1}$. CON: rest = subject values from the sample taken during the CON resting stage; CON:min7 = subject values from the sample taken during the CON exercise stage at minute 7; CON: min10 = subject values from the sample taken during the CON exercise stage at minute 10: CON: min22= subject values from the sample taken during the CON exercise stage at minute 22; CON: min25= subject values from the sample taken during the CON exercise stage at minute 25; CON: min37= subject values from the sample taken during the CON exercise stage at minute 35; mean = the average of the values; SE = the standard error.

Table I-9. Glycerol data for all subjects for CON recovery values

subject	CON:min48	CON: min58	CON:min68	CON:min78	CON:min88	CON:min98
1	53.5	45.5	28.7	35.6	33.6	33.3
2	81.5	64.9	61.8	40.0	28.7	30.3
3	51.4	49.3	40.0	35.7	33.0	37.5
4	66.2	45.2	36.7	37.2	33.7	40.0
5	66.6	49.5	39.0	36.4	35.8	32.0
6	44.8	44.1	36.6	27.8	24.5	25.5
7	48.2	37.7	25.6	24.8	22.4	26.6
8	49.1	50.0	38.6	31.3	26.7	26.5
9	40.7	33.6	39.2	25.1	27.4	36.5
mean	55.8	46.6	38.5	32.7	29.5	32.0
SE	4.3	2.9	3.4	1.9	1.6	1.8

All values in $mg \cdot dl^{-1}$. CON:min48 = subject values from the sample taken during the CON recovery stage at minute 48; CON:min58 = subject values from the sample taken during the CON recovery stage at minute 58; CON:min68 = subject values from the sample taken during the CON recovery stage at minute 68; CON:min78 = subject values from the sample taken during the CON recovery stage at minute 78; CON:min88 = subject values from the sample taken during the CON recovery stage at minute 88; CON:min98 = subject values from the sample taken during the CON recovery stage at minute 98; mean = the average of the values; SE = the standard error.

Table I-10. Glycerol data for all subjects for INT resting and exercise values

subject	INT:rest	INT: min7	INT:min10	INT:min22	INT:min25	INT:min37
1	21.5	15.3	41.6	51.6	48.5	44.8
2	26.9	43.7	58.2	70.1	66.4	86.7
3	28.9	22.7	89.5	108.8	109.5	134.4
4	32.2	36.4	35.4	62.1	59.5	87.4
5	39.9	33.7	62.6	80.6	59.9	87.1
6	41.2	35.0	53.3	64.6	82.2	88.4
7	35.7	29.5	30.7	61.0	79.0	99.8
8	40.5	34.3	41.7	60.2	50.5	59.0
9	29.9	36.7	44.0	72.4	55.7	74.3
mean	33.0	31.9	50.8	70.2	67.9	84.7
SE	2.3	2.8	6.0	5.6	6.5	8.4

All values in mg·dl^{-1}. INT: rest = subject values from the sample taken during the INT resting stage; INT:min7 = subject values from the sample taken during the INT exercise stage at minute 7; INT: min10 = subject values from the sample taken during the INT exercise stage at minute 10: INT: min22= subject values from the sample taken during the INT exercise stage at minute 22; INT: min25= subject values from the sample taken during the INT exercise stage at minute 25; INT: min37= subject values from the sample taken during the INT exercise stage at minute 35; mean = the average of the values; SE = the standard error.

Table I-11. Glycerol data for all subjects for INT recovery values

subject	INT:min48	INT: min58	INT:min68	INT:min78	INT:min88	INT:min98
1	49.7	42.8	30.5	26.1	20.3	20.5
2	90.8	95.4	62.1	50.5	36.5	40.7
3	142.7	95.6	89.0	79.0	66.9	40.5
4	85.6	74.1	62.3	47.0	41.2	33.5
5	65.0	55.4	49.3	38.2	37.4	58.4
6	76.0	69.0	62.3	62.3	59.1	45.7
7	65.7	55.0	42.1	38.6	29.8	34.6
8	61.8	88.0	55.4	49.3	29.9	38.5
9	45.6	36.3	29.5	30.8	46.1	35.0
mean	75.9	68.0	53.6	46.9	40.8	38.6
SE	9.7	7.4	6.2	5.4	4.9	3.4

All values in mg$\cdot$dl^{-1}. INT:min48 = subject values from the sample taken during the INT recovery stage at minute 48; INT:min58 = subject values from the sample taken during the INT recovery stage at minute 58; INT:min68 = subject values from the sample taken during the INT recovery stage at minute 68; INT:min78 = subject values from the sample taken during the INT recovery stage at minute 78; INT:min88 = subject values from the sample taken during the INT recovery stage at minute 88; INT:min98 = subject values from the sample taken during the INT recovery stage at minute 98; mean = the average of the values; SE = the standard error.

Table I-12. Blood glucose data for all subjects for CON resting and exercise values

subject	CON:rest	CON: min7	CON:min10	CON:min22	CON:min25	CON:min37
1	92	93	92	88	89	88
2	79	90	92	86	97	87
3	89	88	87	72	81	82
4	86	95	91	88	93	96
5	88	94	97	89	91	89
6	92	108	103	100	100	103
7	85	92	81	79	80	84
8	90	91	97	89	97	98
9	88	94	95	98	99	101
mean	88	94	93	88	92	92
SE	1	2	2	3	2	3

All values in $mg \cdot dl^{-1}$. CON: rest = subject values from the sample taken during the CON resting stage; CON:min7 = subject values from the sample taken during the CON exercise stage at minute 7; CON: min10 = subject values from the sample taken during the CON exercise stage at minute 10: CON: min22= subject values from the sample taken during the CON exercise stage at minute 22; CON: min25= subject values from the sample taken during the CON exercise stage at minute 25; CON: min37= subject values from the sample taken during the CON exercise stage at minute 35; mean = the average of the values; SE = the standard error.

Table I-13. Blood glucose data for all subjects for CON recovery values

subject	CON:min48	CON: min58	CON:min68	CON:min78	CON:min88	CON:min98
1	89	87	95	87	89	92
2	79	77	82	82	79	81
3	83	98	97	93	93	92
4	88	88	90	92	92	90
5	93	93	90	97	91	95
6	113	113	105	107	102	99
7	84	82	79	77	83	82
8	92	87	84	81	79	81
9	99	101	99	95	94	91
mean	91	92	91	90	89	89
SE	3	4	3	3	3	2

All values in $mg \cdot dl^{-1}$. CON:min48 = subject values from the sample taken during the CON recovery stage at minute 48; CON:min58 = subject values from the sample taken during the CON recovery stage at minute 58; CON:min68 = subject values from the sample taken during the CON recovery stage at minute 68; CON:min78 = subject values from the sample taken during the CON recovery stage at minute 78; CON:min88 = subject values from the sample taken during the CON recovery stage at minute 88; CON:min98 = subject values from the sample taken during the CON recovery stage at minute 98; mean = the average of the values; SE = the standard error.

Table I-14. Blood glucose data for all subjects for INT resting and exercise values

subject	INT:rest	INT: min7	INT:min10	INT:min22	INT:min25	INT:min37
1	99	98	101	104	114	129
2	77	82	86	92	101	110
3	92	91	91	91	97	110
4	81	92	90	100	108	128
5	92	99	96	96	95	108
6	95	102	103	115	118	124
7	97	98	91	89	94	102
8	81	90	90	113	123	132
9	84	91	98	107	111	110
mean	89	99	94	101	107	117
SE	3	2	2	3	4	4

All values in $mg \cdot dl^{-1}$. INT: rest = subject values from the sample taken during the INT resting stage; INT:min7 = subject values from the sample taken during the INT exercise stage at minute 7; INT: min10 = subject values from the sample taken during the INT exercise stage at minute 10: INT: min22= subject values from the sample taken during the INT exercise stage at minute 22; INT: min25= subject values from the sample taken during the INT exercise stage at minute 25; INT: min37= subject values from the sample taken during the INT exercise stage at minute 35; mean = the average of the values; SE = the standard error.

Table I-15. Blood glucose data for all subjects for INT recovery values

subject	INT:min48	INT: min58	INT:min68	INT:min78	INT:min88	INT:min98
1	119	107	108	102	99	107
2	106	101	91	95	92	87
3	112	110	106	103	97	94
4	129	116	102	87	82	85
5	102	101	98	94	84	89
6	124	124	119	110	102	101
7	99	85	76	75	73	79
8	120	101	85	70	64	56
9	99	91	85	82	79	82
mean	112	104	97	91	86	87
SE	4	4	5	4	4	5

All values in $mg \cdot dl^{-1}$. INT:min48 = subject values from the sample taken during the INT recovery stage at minute 48; INT:min58 = subject values from the sample taken during the INT recovery stage at minute 58; INT:min68 = subject values from the sample taken during the INT recovery stage at minute 68; INT:min78 = subject values from the sample taken during the INT recovery stage at minute 78; INT:min88 = subject values from the sample taken during the INT recovery stage at minute 88; INT:min98 = subject values from the sample taken during the INT recovery stage at minute 98; mean = the average of the values; SE = the standard error.

Table I-16. Blood lactate data for all subjects for CON resting and exercise values

subject	CON:rest	CON: min7	CON:min10	CON:min22	CON:min25	CON:min37
1	0.9	2.9	3.8	4.3	4.4	3.4
2	1.2	3.2	3.5	4.1	4.2	4.4
3	1.0	4.1	5.1	6.1	6.4	4.9
4	1.0	3.4	4.8	5.4	5.5	4.6
5	0.9	2.0	2.9	3.4	3.5	2.6
6	1.3	3.0	3.7	3.7	3.8	3.3
7	0.8	2.8	3.6	4.0	4.2	3.4
8	0.8	2.3	3.4	4.1	4.3	4.0
mean	1.0	3.0	3.9	4.4	4.5	3.8
SE	0.1	0.2	0.3	0.3	0.3	0.3

All values in mg·dl^{-1}. CON: rest = subject values from the sample taken during the CON resting stage; CON:min7 = subject values from the sample taken during the CON exercise stage at minute 7; CON: min10 = subject values from the sample taken during the CON exercise stage at minute 10: CON: min22= subject values from the sample taken during the CON exercise stage at minute 22; CON: min25= subject values from the sample taken during the CON exercise stage at minute 25; CON: min37= subject values from the sample taken during the CON exercise stage at minute 35; mean = the average of the values; SE = the standard error.

Table I-17. Blood lactate data for all subjects for CON recovery values

subject	CON:min48	CON: min58	CON:min68	CON:min78	CON:min88	CON:min98
1	2.0	1.4	1.1	0.9	0.9	0.8
2	3.3	2.6	1.9	1.4	1.1	1.0
3	3.4	2.5	1.9	1.5	1.2	1.0
4	2.7	1.9	1.3	1.0	0.8	0.8
5	1.6	1.2	0.9	0.9	0.7	0.7
6	2.3	1.7	1.5	1.3	1.3	1.2
7	1.8	1.3	0.9	0.8	0.6	0.6
8	2.7	1.9	1.3	1.2	1.0	1.0
mean	2.5	1.8	1.4	1.1	1.0	0.9
SE	0.2	0.2	0.1	0.1	0.1	0.1

All values in $mg \cdot dl^{-1}$. CON:min48 = subject values from the sample taken during the CON recovery stage at minute 48; CON:min58 = subject values from the sample taken during the CON recovery stage at minute 58; CON:min68 = subject values from the sample taken during the CON recovery stage at minute 68; CON:min78 = subject values from the sample taken during the CON recovery stage at minute 78; CON:min88 = subject values from the sample taken during the CON recovery stage at minute 88; CON:min98 = subject values from the sample taken during the CON recovery stage at minute 98; mean = the average of the values; SE = the standard error.

Table I-18. Blood lactate data for all subjects for INT resting and exercise values

subject	INT:rest	INT: min7	INT:min10	INT:min22	INT:min25	INT:min37
1	0.7	3.4	6.0	8.1	8.3	9.6
2	1.0	4.1	6.1	9.7	9.8	9.5
3	1.2	4.9	8.1	11.5	12.4	14.6
4	1.3	4.3	6.8	10.7	11.3	13.2
5	0.7	3.1	6.0	9.0	9.3	10.0
6	1.2	4.6	6.9	12.1	12.7	15.1
7	1.1	4.9	8.4	11.6	11.8	12.7
8	0.9	3.9	5.6	11.2	11.1	12.8
mean	1.0	4.2	6.7	10.5	10.8	12.2
SE	0.1	0.2	0.4	0.5	0.6	0.8

All values in $mg{\cdot}dl^{-1}$. INT: rest = subject values from the sample taken during the INT resting stage; INT:min7 = subject values from the sample taken during the INT exercise stage at minute 7; INT: min10 = subject values from the sample taken during the INT exercise stage at minute 10: INT: min22= subject values from the sample taken during the INT exercise stage at minute 22; INT: min25= subject values from the sample taken during the INT exercise stage at minute 25; INT: min37= subject values from the sample taken during the INT exercise stage at minute 35; mean = the average of the values; SE = the standard error.

Table I-19. Blood lactate data for all subjects for INT recovery values

subject	INT:min48	INT: min58	INT:min68	INT:min78	INT:min88	INT:min98
1	6.8	4.7	3.4	2.5	2.1	1.9
2	5.9	4.7	3.7	2.9	2.4	2.0
3	13.8	9.9	9.1	6.5	5.5	4.2
4	12.0	9.6	7.5	5.9	4.7	3.8
5	7.9	5.2	3.4	2.6	2.0	1.7
6	13.6	10.7	8.4	5.9	4.7	4.5
7	10.2	7.7	4.0	3.3	2.6	2.1
8	10.4	9.1	7.4	5.9	5.0	4.2
mean	10.1	7.7	5.9	4.4	3.6	3.1
SE	1.1	0.9	0.9	0.6	0.5	0.4

All values in $mg \cdot dl^{-1}$. INT:min48 = subject values from the sample taken during the INT recovery stage at minute 48; INT:min58 = subject values from the sample taken during the INT recovery stage at minute 58; INT:min68 = subject values from the sample taken during the INT recovery stage at minute 68; INT:min78 = subject values from the sample taken during the INT recovery stage at minute 78; INT:min88 = subject values from the sample taken during the INT recovery stage at minute 88; INT:min98 = subject values from the sample taken during the INT recovery stage at minute 98; mean = the average of the values; SE = the standard error.